

**CONSERVED EPITOPES IN *PLASMODIUM FALCIPARUM* AND INFLUENZA  
A VIRUS AS TARGETS FOR VIRUS-VECTORED IMMUNIZATION**

By:  
Cailin Deal

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## Abstract

The development of vaccines against infectious diseases has been highly successful. However, while effective vaccines have been licensed against many diseases such as smallpox, polio, measles, rubella, mumps, varicella, diphtheria, tetanus and pertussis, there are still a number of infectious diseases that continue to wreak havoc on public health. Many attempts have been made to create vaccines against intractable pathogens, such as HIV, malaria, and influenza A virus (IAV), but the construction of vaccines that induce broadly protective immunity against these diseases has proven difficult. This necessitates further research into conserved epitopes and new vaccine approaches.

In this study, I investigate the role of a highly conserved epitope of influenza A virus, the M2 extracellular domain (M2e), in the viral life cycle; this region has become a major molecular target for universal IAV vaccines. Using *in vitro* transcomplementation assays with cell lines stably expressing M2e mutants, we determined that this region of the M2 protein is tolerable of mutations since directed alanine mutagenesis nor deletion of several amino acids attenuated IAV replication. This suggests that this portion of M2e is not required for function and that alternative reasons exist for the high conservation.

In addition, the use of viral vectors for vaccines was investigated. Adenovirus (Ad) is a highly immunogenic viral vector. Recombinant Ads that display either the M2e or conserved HA2 alpha helix (HA2A) of IAV in hexon hypervariable regions were examined as potential universal influenza A vaccines. While these recombinant Ads were successfully created, they were not immunogenic in mice. Induction of effective immune responses by conventional vaccination methods is uncertain, especially for pathogens such as malaria and HIV. As an alternative to relying on an individuals' immune system to mount a protective response, adeno-associated virus (AAV) was used to express previously characterized human broadly neutralizing monoclonal antibodies to the *Plasmodium falciparum* circumsporozoite protein (CSP) as a

transgene. This ‘vectored immunoprophylaxis’ (VIP) approach allowed for rapid and high sustained expression of monoclonal antibodies to CSP that were capable of protecting mice from stringent sporozoite challenge, making this a viable approach for malaria prevention.

**Adviser:**

Gary Ketner

**Readers:**

Andrew Pekosz

William Guggino

Anna Durbin

**Alternates:**

Fidel Zavala

Juergen Bosch

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## **Chapter One:**

### **Broadly protective universal influenza vaccines based on the conserved HA2 and M2 extracellular domain**

Each year, epidemics of influenza caused by either influenza A (IAV) or B viruses circulate through the human population causing significant morbidity and mortality, responsible for 3-5 million clinical infections and 250,000 – 500,000 deaths [1, 2]. More than 90% of influenza associated deaths occur among adults 65 or older [3]. In the United States, an estimated 6-20% of the population is infected with influenza each year [4]. The number of deaths that occur each year due to this respiratory virus varies considerably depending upon circulating strains and vaccine efficacy [3]. Currently, H1N1 and H3N2 of IAV and both strains of influenza B viruses are responsible for seasonal outbreaks.

Seasonal outbreaks in most parts of the world coincide with colder weather. This is in part correlated to an increase in individuals crowding indoors during cold seasons, as the virus can be spread by aerosolized droplets from coughing or sneezing, along with the decrease in temperature and humidity that allows the virus to survive in the environment for longer periods of time [5]. Most seasonal outbreaks are due to antigenic drift of influenza viruses, a process where amino acid changes are selected for that enable the virus to escape neutralizing antibodies [6]. However, periodically, antigenic shift occurs, where gene reassortment involving human, swine and/or avian influenza viruses yield in an antigenically distinct virus, usually resulting in the introduction of a new hemagglutinin (HA or H) gene into the population [6]. This process resulted in the 2009 H1N1 pandemic, where a triple reassortment of avian, swine and human viruses created a novel strain of influenza that led to a significant increase in morbidity and mortality.

The recurrences of highly pathogenic strains of influenza have been spread throughout history. A novel zoonotic influenza virus strain emerged with a genotype that resulted from genetic reassortment and multiple point mutations in 1918 [7-9]. This resulted in a highly transmissible and virulent virus that led to the “Spanish” flu outbreak. It infected one-third of the population worldwide in a year and exhibited a case fatality rate of approximately 10%, killing 40

million people [8, 10], marking the deadliest influenza pandemic to date. In addition to the severe death toll, the 1918 H1N1 virus was unique in exacting the greatest toll on young healthy adults rather than infants and the elderly, in contrast to seasonal epidemics. While there are many possible explanations as to this age distribution, one suggests that those over 65 could have acquired cross-reactive influenza-specific antibodies prior to the 1918 pandemic, leaving those between age 15 and 45 more susceptible [9].

Novel serotypes of influenza continue to arise due to genetic reassortment with existing human strains. In 1957, reassortment between the 1918 H1N1 and an avian strain yielded a novel H2N2 [10]. The acquisition of a novel HA and neuraminidase (NA or N) in a naïve population led to 40% of people becoming infected with approximately 50% of infections leading to severe disease as a result of secondary bacterial pneumonia [11]. Subsequently, in 1968, another reassortment occurred between human and avian viruses resulting in an H3N2 strain. The novel H3 allowed for the virus to be effectively transmitted within a population however, preexisting immunity to the H3 likely limited morbidity and mortality [10, 12]. H1N1 strains were reintroduced into the population in 1977 without causing a major pandemic due to immunity that was already developed against both HA and NA [10]. Direct bird to human transmission resulted in emergence of another novel influenza strain, an H5N1, in Hong Kong in 1997 [13]. While it has a 50% mortality rate, it exhibits poor direct human to human transmission and did not result in a pandemic [14]. Currently, most H5N1 infections have been due to direct contact with infected domestic poultry. The first 21<sup>st</sup> century pandemic emerged in 2009 as a novel H1N1 that was a product of a recombination event with swine, avian and human viruses [15]. While H1N1 viruses have been circulating in the population for decades, the antigenic properties of this strain was sufficiently unique that most individuals lacked effective preexisting immunity and were susceptible to infection [16, 17]. More recently, an H7N9 avian flu has emerged in southern China, infecting approximately 140 people and killing 45. However, the virus has not achieved

effective human to human transmission as 70% of cases have resulted from direct contact with infected poultry [18].

Pandemic viruses are rare but seasonal epidemics occur yearly. While an influenza vaccine is licensed and updated yearly, it is only effective against homologous strains of influenza and provides very little cross-protection from antigenic drift viruses. This not only results in morbidity and mortality but also has a significant economical impact. It was estimated that \$87.1 billion are lost annually due to the lack of productivity and medical costs associated with influenza illness [4]. Government and private institutions spend a significant amount of money on surveillance, basic research and public health education, in addition to the \$2-4 billion that is spent annually on vaccine production [19]. Thus, while there is a licensed influenza vaccine, it does not offer sufficient protection for highly susceptible populations or to the general population during years of significant antigenic diversity [20, 21], to prevent the high economic cost and public health burden that accrues during seasonal influenza. An effective cross-protective vaccine would reduce morbidity, mortality and influenza-associated economic costs. It is particularly important to have a vaccine that offers effective protection to young infants and the elderly who are the most susceptible populations to influenza infection and subsequent complications [22, 23]. This necessitates more in-depth investigations into basic influenza virology, the immune response and host correlates of protection to rationally design a ‘universal’ vaccine, capable of inducing effective cross-protective immune responses.

### **Influenza virology**

To develop an effective vaccine that is capable of inducing a robust humoral and cellular immune response against all influenza virus subtypes, a basic understanding of influenza virology is imperative. Influenza virus is an RNA virus belonging to the *Orthomyxoviridae* family. It has an 8 segment negative sense RNA genome [24] that is packaged into a primarily spherical lipid-

envelope (Figure 1A), although elongated filamentous particles are found in influenza strains isolated from humans [25-27]. Five genome segments encode for a single viral protein, while segments encoding PB1, NA and M have more than one open reading frame leading to multiple mRNAs produced by splicing or frame shift [28-30]. Eleven viral proteins have been identified thus far in IAV although not all strains produce the PB1-F2 protein [30].

In humans, influenza is transmitted primarily through aerosolized respiratory droplets [31] and infection starts when the HA on the virion surface binds to its receptor, a cell surface sialic acid (SA) [32]. Avian influenza viruses preferentially bind SA that is linked to galactose via a  $\alpha 2,3$  linkage ( $\alpha 2,3$  SA) which can be found in the gut whereas human strains bind preferentially to  $\alpha 2,6$  linked SA ( $\alpha 2,6$  SA) found in the respiratory tract [33]. However, influenza viruses that infect swine have more diverse receptor specificity by being able to bind either  $\alpha 2,3$  or  $\alpha 2,6$  SA [33-35]. The ability to bind different sialyloligosaccharides is important for cross-species transmission of the virus as viruses with this property are able to infect many different hosts and cause more systemic infections [35, 36].

Upon receptor binding, influenza is internalized by clathrin-mediated endocytosis [37] and acidification of the late endosome triggers conformational changes in HA that produces a fusogenic peptide [38]; this results in fusion of the viral membrane with the endosomal membrane [39]. Concurrently, the integral ion channel protein, M2, pumps  $H^+$  ions into the virion interior [40-42]. The acidification of the interior of the virus prompts M1 dissociation from viral ribonucleoproteins (RNP). These RNPs consist of the viral RNA segments, the viral RNA-dependent RNA polymerase (RdRp) comprised of PB1, PB2 and PA, and the nucleoprotein (NP) [43, 44]. Dissociation from M1 allows for the RNPs to be imported into the nucleus via host importin complexes and a nuclear localization signal (NLS) located in the NP protein [43, 45]. Influenza is one of the few RNA-containing viruses that replicate within the nucleus due to the requirement for host splicing machinery, 5' 7-methylguanosine caps from host pre-mRNAs and

potentially, to sequester RNA away from pathogen recognition receptors (PRR) that can activate the immune response [46].

Once inside the nucleus, PA, PB1 and PB2 form the RdRp. While the PB1 protein contains the catalytic subunit of the RNA polymerase, allowing for the addition of nucleotides during RNA chain elongation [47], it also has endonuclease activity necessary for generation of a capped primer. Replication is initiated upon PB1-mediated cap snatching of 5' caps from cellular mRNA which acts as a primer for viral mRNA synthesis [46]. A polyadenosine tail is added to the 3' end of the genome due to RdRp stuttering and is important for nuclear export, translation and mRNA stability. Positive-sense RNA serves as a template to synthesize negative-sense RNA which will be exported from the nucleus upon association with M1 and the viral nuclear export protein (NEP/NS2) [46]. These negative-sense RNAs will eventually be incorporated into new virions to continue the viral life cycle.

Inside the nucleus, the M, PB1 and NA mRNA segments are subject to cellular splicing by the splice factor SF2/ASF [48]. Splicing is tightly regulated as protein needs to be produced from both unspliced and spliced mRNA. Many control mechanisms for differential splicing exist including the rate of nuclear export and access to the splice site. For example, both M1 and M2 are synthesized from the M genomic RNA segment by differential splicing. The three spliced mRNA transcripts encoded by the M segment share the same 3' splice site but have alternative 5' splice sites. At early times during infection, the strong 5' splice site on the M segment is used, resulting in M3-mRNA [49]. Once a sufficient amount of the RdRp complex has been synthesized, the polymerase proteins bind to the 5' terminal M1-specific sequences in mRNAs, blocking access to the 5' M3-mRNA splice site; this allows for unspliced M1-mRNA to be synthesized [50]. As the polymerase complex moves down the mRNA, it blocks the 5' M1-mRNA splice site, allowing for the cellular splicing machinery to switch to the weaker 5' M2-splice site to create M2-mRNA. However, the splice site for M2 is suboptimal and its recognition



depends upon the SF2/ASF splice factor binding to a splice enhancer at the 3' end of the mRNA [48, 50]. This demonstrates the complexity of the viral and cellular interactions necessary for influenza virus replication.

Following splicing, mRNA's are transported to the cytoplasm where viral membrane proteins, HA, NA and M2, are translated by ribosomes bound to the endoplasmic reticulum (ER) [46]. These proteins then enter the secretory pathway where, inside the ER, both HA and NA are glycosylated, HA is assembled into a trimer and NA and M2 form tetramers. Cysteine residues on HA and M2 are palmitoylated in the *cis*-Golgi network [51] and M2 uses its ion channel activity in the *trans*-Golgi to stabilize the HA protein and prevent conformational rearrangement [52]. From the secretory pathway, HA, NA and M2 are targeted to the host cell membrane. Both HA and NA have signal sequences in their transmembrane domain that target them to lipid rafts that are rich in sphingolipids and cholesterol [46]. Most of M2 is selectively excluded from lipid rafts and a sorting signal for M2 has not been characterized. It is suggested that M2 might bind to cholesterol which would target it to the periphery of lipid rafts [53, 54].

Relatively little is known about how viral proteins other than HA and NA are incorporated into virions. Virus budding has been shown to preferentially occur at lipid rafts where HA and NA are localized [55, 56]. M1 has been postulated to be recruited into the virion by crosslinking the cytoplasmic tails of HA and NA [57, 58]. Experimental evidence demonstrates that the cytoplasmic tail of M2 helps to incorporate RNPs [59], either by direct binding to NP or through interactions with M1 although the exact packaging mechanism of RNPs is also poorly understood. A fully infectious influenza virion requires a full complement of RNA segments and multiple pieces of evidence have demonstrated that the virus selectively packages eight different vRNPs into virus particles. The cytoplasmic tail of M2 has been shown to be required for efficient vRNP incorporation of infectious virus particles [60-62]. It is also still unknown how M2 is incorporated into the virion since it is excluded from sites of viral budding

[56]. Currently it is speculated that binding to M1 [60] may recruit M2 to the periphery of lipid rafts where it would be able to bind cholesterol [53, 63, 64] and become incorporated into the virion.

The initiation of budding requires an outward curvature of the plasma membrane and experimental evidence suggest that this process is started with HA and NA [65]. These glycoproteins are targeted to lipid raft domains and then recruit M1, NP and RNPs [56]. M2 is recruited to the site of budding by M1 [64]. In addition to the positive membrane curvature asserted by HA, NA and M1 [65, 66], M2 exerts more positive membrane curvature on the neck of the budding virion by inserting its amphipathic helix into the membrane, thus mediating membrane scission and release [53]. This hypothesis has not been fully proven.

The final step in the influenza life cycle is the release of the virion from the site of budding. Influenza virus has to be actively released from the host cell because HA is able to anchor the virus to the cell by binding to its sialic acid receptor [46]. Here, the enzymatic activity of NA is required to remove the sialic acid from the host cell membrane thus releasing the virus [67, 68]. It also cleaves the sialic acid off viral glycoproteins to prevent virus aggregation. The influenza virus is not fully infectious until the immature HA0 that is present on the viral surface is cleaved by host proteases into HA1/HA2 [69, 70]. This exposes the binding site on the head of HA1 that recognizes SA. The ability of multiple different types of proteases to cleave HA0 is dependent upon the composition of the proteolytic site and determines the pathogenicity of the virus [71]. Influenza A viruses with a multibasic cleavage site containing several lysines and/or arginines, are more virulent and induce more systemic infections as many different proteases are capable of cleaving HA0. Viruses with a monobasic HA cleavage site are only capable of initiating infection in the respiratory tract where extracellular trypsin-like proteases are present [71].

## **Innate immune response**

Replication of influenza virus does not go unnoticed in the host. After bypassing physical barriers to infiltrate the respiratory tract, influenza virus infection is sensed by infected cells, usually respiratory epithelial cells, via PRR that include toll like receptors (TLR), retinoic acid inducible gene-1 (RIG-I) and the NOD-like receptor family pyrin domain containing 3 (NLRP3) [72]. These all recognize different forms of viral RNA. TLR7 binds single-stranded viral RNA whereas TLR3 and RIG-I recognize double-stranded viral RNA [73-75]. Recognition by these receptors leads to the production of proinflammatory cytokines and type I interferons (IFNs). IFNs have a strong antiviral activity by inhibiting protein synthesis in host cells and limiting virus replication. IFN- $\beta$  is produced first by signaling through the PRRs and, via a positive feedback loop, leads to the expression of IFN- $\alpha$  and more IFN- $\beta$  [76]. These type I IFNs also induce interferon stimulating genes (ISG), such as the myxovirus (MX) gene which encodes the antiviral MxA protein, through the JAK/STAT signaling pathway [77, 78] and have been shown to stimulate dendritic cells (DCs), a professional antigen-presenting cell (APC), to enhance presentation of antigen on CD4<sup>+</sup> and CD8<sup>+</sup> cells through major histocompatibility complex II (MHCII) and MHCI, respectively. During this process, NLRP3, a component of the inflammasome, a cytoplasmic complex associated with immunity to influenza virus, is activated by the influenza M2 ion channel activity [79]. This component induces the induction of Th17 cells and expansion of CD4<sup>+</sup> T cells.

Once the virus reaches the alveoli, alveolar macrophages are activated. This subset of highly effective APC phagocytoses influenza-infected cells [80, 81]. However, while the activation of alveolar macrophages do limit viral spread, they produce nitric oxide synthase 2 (NOS2) and tumor necrosis factor alpha (TNF- $\alpha$ ) which causes immune mediated pathology [82,

83]. This highlights the delicate balance that is required of the immune response. Highly pathogenic avian influenza (HPAI) viruses are lethal because they are able to infect blood-derived macrophages, leading to the production of large quantities of pro-inflammatory cytokines which causes immunopathology [84].

Natural killer (NK) cells are also an important arm of the innate immune response as they are able to recognize infected cells through their cytotoxicity receptor NKp44 and NKp46 which, upon binding, will trigger NK cells to lyse the infected cells [85]. A subset of NK cell called invariant NKT cells are thought to stimulate the induction of cellular immunity and regulate infection induced pathology [86].

DCs are a major component of the host innate immune system and are situated close to the airway epithelium and basal membrane in order to monitor the airway lumen for infection via their dendrites. Upon detection of virus, DCs phagocytize opsonized virions and apoptotic bodies released from infected cells. While these cells can become infected by influenza virus, it does not result in viral replication or immune-mediated pathology but instead, the DC migrates to the draining lymph node via the lymphatic system and presents influenza viral antigens to CD4+ or CD8+ T cells to initiate the adaptive immune response [87].

While the immune response is capable of sensing influenza virus infection, the virus itself expresses proteins that can inhibit the induction of the innate immune response. In particular, the pathways that stimulate type I IFN production are targeted by the NS1 protein which interferes with PRR that detect viral RNA [88]. This IFN antagonist also inhibits post-transcriptional production of IFN by interfering with mRNA processing factors and export machinery [89-91]. Finally, NS1 has also been shown to disrupt IFN stimulated genes [88, 92]. These functions of NS1 demonstrate how the virus has evolved to evade immune detection and ultimately contribute to the pathogenicity of influenza.

## **Adaptive immune response**

The innate immune response is responsible for controlling and containing the viral infection until the adaptive immune response can be activated. This second line of defense consists of two main aspects: cellular immunity mediated by T cells and humoral immunity through the action of virus-specific antibodies.

Upon infection with influenza virus, APCs such as DCs, phagocytose or endocytose exogenous antigen which is then processed into peptides to be presented on MHCI for cytotoxic CD8+ T cells or on MHCII for CD4+ T helper cells (Th). Importantly, CD4+ Th cells initially are considered Th0 cells and can be further developed to promote specific responses: Th1 or Th2 [93]. Th1 cells stimulate production of IgG2A antibody and are mainly involved in cellular immune responses and promoting the proliferation of CD8+ cytotoxic T cells whereas Th2 cells stimulate production of IgG1, IgA and IgE antibody production, thus promoting B cell responses [94]. In addition to the multiple types of CD4+ T cells, there are regulator T cells (Treg) and Th17 cells. Generally, Treg cells are associated with anti-inflammatory responses while Th17 induce proinflammatory responses. It is through the use of these cells, and others that the immune response attempts to create a balance of proinflammatory and anti-inflammatory responses to kill the invading pathogen but also prevent immune-mediated pathology.

CD8+ T cells are an important aspect of the cell mediated immune response. Upon recognition of antigenic peptide presented on MHCI, IFN- $\gamma$  is released which has antiviral activity and activates macrophages. In addition to the release of a potent type II IFN, CD8+ T cells have lytic activity mediated by the release of perforin and granzymes A (GrA) and GrB and can also kill virally infected cells directly through Fas/FasL interactions, leading to the activation of caspases and apoptosis.

The other arm of the adaptive immune response is humoral immunity, characterized by antibody produced by B cells. While T cells recognize linear epitopes, B cells recognize antigen in its native form. The principal function of B cells is to make antibodies that are membrane bound or secreted. Antibodies specific for influenza antigens are then used to identify and neutralize influenza virions or influenza infected cells. Most antibody-mediated protection is due to secretory IgA (sIgA) and IgG [95]. SIgA is able to be transported across mucosal epithelium of the upper respiratory tract and are the first antibody barrier to influenza viruses. Serum IgA is produced rapidly after influenza virus infection and the presence of these antibodies is indicative of a recent influenza infection [96, 97]. IgG is able to transude from serum into the respiratory tract by diffusion and has been shown to be primarily responsible for protection of the lower respiratory tract as it affords long-lived protection [98]. However, not all antibody-mediated protection is due to those two types of immunoglobulins. IgM antibodies are able to initiate complement, a biochemical cascade of the innate immune system, to neutralize influenza virus and their presence indicates a primary infection as it is the first immunoglobulin expressed by mature B cells [99, 100].

There are multiple different ways that antibody is capable of providing protection against a virion. They can inhibit assembly of progeny virus, prevent uncoating or aggregation of virions, destabilize the virion structure or inhibit an important viral function through a signal transduction cascade. For example, upon dimeric IgA binding to a polymeric Ig receptor expressed on the basolateral surface of epithelial cells, it becomes transcytosed to the apical surface where the poly-Ig receptor is cleaved. SIgA is then released to prevent infection; this mode of intracellular neutralization has been noted in anti-HA IgA antibodies [95]. Antibodies specific for the variable globular head of HA are able to bind and prevent attachment of influenza to SA receptors on the cell [101-103], thus preventing infection whereas antibodies that bind to the more highly conserved stem region of HA prevent the conformational changes necessary for

fusion [104-107]. Along with the globular HA head, NA-specific antibodies are in abundance and correlate with protective immunity [108]. These antibodies do not neutralize the virus, unlike HA but instead inhibit the enzymatic activity of NA that is required for release of the budding virus which causes aggregation of virus particles on the infected cell surface [108, 109]. Not all antibodies bind directly to the virus but can also coat infected cells to eliminate them through ADCC, mediated by recognition of the antibody Fc receptor by NK cells. Antibody-coated infected cells can also be opsonized by macrophages or activate the classical complement pathway [100, 110-112]. It is thought that M2- and NP-specific antibodies function in one of these ways as they are both protective but non-neutralizing.

Once the viral infection is cleared, residual influenza-specific B and T cells are found in lymphoid organs and in circulation and comprise the memory immune response. This population is maintained under DC and T cells producing IL-17 and can reactivate quickly upon subsequent influenza virus infections [113]. Most human memory responses are against the NP, M1 and PA proteins [114-117]. Due to the high conservation of these proteins, the memory response is highly cross-reactive between different influenza A subtypes. However, epitopes recognized by virus-specific CTL are also under selective pressure and the virus has the capacity to overcome functional constraints through compensatory mutations in order to evade T cell immunity.

### **Licensed influenza A vaccines**

There are currently two different types of licensed influenza vaccines: an inactivated and a live attenuated influenza virus (LAIV). Both vaccines have a trivalent or a quadrivalent version with the trivalent currently being composed of two different IAV strains: an H1N1 and an H3N2 along with one of the two influenza B strains while the quadrivalent incorporates the same IAV strains but also both influenza B strains [118, 119]. These vaccines are updated yearly to antigenically match strains that the WHO global surveillance predicts will predominantly

circulate in the upcoming flu season [120, 121]. Antibodies are induced against HA and NA in which there is documented protective efficacy.

Inactivated influenza vaccines have been extensively used for decades; the first commercial vaccine using whole inactivated influenza virus was approved for use in the United States in 1945 [122, 123]. This vaccine consists of purified virus that has been chemically inactivated with formalin or  $\beta$ -propiolactone and in most formulations, is also treated with detergent to produce soluble forms of the viral surface antigens. Each of the IAV subtypes are adapted to grow in embryonated eggs or are a reassortant containing the HA and NA of strains included in the vaccine with the rest of the viral genome from the A/Puerto Rico/8/34 (PR8; H1N1) virus [124]. This virus allows for high growth capacity in eggs to increase vaccine production. However, HA and NA can affect the growth characteristics of reassortant viruses such that certain combinations are not as well-adapted in eggs [125].

While the inactivated vaccine has provided >59% efficacy in the past 12 flu seasons in adults 18 to 65 years old [21], there are still some inherent failures. First, the duration of the protective immunity induced by the inactivated vaccine is short, with levels of antibody decreasing by 75% over an 8-month period in some patients [126]. Additionally, the intramuscular route of injection of this vaccine fails to induce a local mucosal immune response, where influenza infection would occur, and also does not induce robust cellular immunity [127-129]. The inactivated vaccine also exhibits variable efficacy and is unable to protect against antigenic shift and drift viruses [120].

The development of the LAIV aimed to address several of the issues associated with the inactivated vaccines. While the LAIV is a live virus, delivered by a nasal spray, it has been cold-adapted such that it can only replicate at 25°C, the approximate temperature of the nasal passage, is temperature sensitive and is unable to replicate at 37° or 39°C which are temperatures



associated with the lower respiratory tract. The LAIV virus is further attenuated to contain other mutations that prevent it from producing flu-like illness. For the seasonal vaccines, the HA and NA of the influenza strains to be included in the vaccine is reassorted with the internal proteins from the master donor attenuated virus (MDV): A/Ann Arbor/6/60 and B/Ann Arbor 1/66. The MDV was initially developed by serial passage at sequentially lower temperatures, leading to the acquired cold adapted, temperature sensitive and attenuated phenotypes due to multiple mutations in the internal viral proteins [130]. This vaccine exhibits higher efficacy in children as compared to the inactivated [118, 131] and intranasal delivery allows for the production of serum IgG and mucosal IgA antibody that are associated with protection at mucosal surfaces [132-134]. In addition, this vaccine induces a robust cellular immune response that is more cross-protective than antibodies and may contribute to protection against clinical symptoms [135]. However, this vaccine also has its weaknesses in that it is less effective in adults and is not approved for use in people over 50, a subpopulation that is highly susceptible to flu infection and complications [131, 136]. This necessitates further understanding of the immune correlates of protection in order to develop new vaccines capable of offering protection to highly susceptible populations.

Currently, the gold standard for evaluation of immunogenicity of influenza vaccines is the HA serum antibody titer measured by the HA inhibition (HI) assay, with a titer greater than 40 being considered protective [137, 138]. A dose of 15µg of HA is sufficient to induce this level of antibody but only in adults who were previously exposed to an influenza virus of the same subtype. It takes a higher dose of vaccine or multiple doses in elderly or unprimed individuals [139, 140]. Flu viruses have evolved since the creation of the HI assay and new influenza viruses, such as H5N1 and H7N7, pose a significant public health threat. In preclinical animal studies for H5N1 vaccines, it has been difficult to detect H5 specific HI antibodies, even in H5N1-infected humans [141, 142]. While influenza vaccines have been evaluated by the HI assay historically, there is an enormous need to develop alternative correlates of protection.

Research has been ongoing into the development of new influenza vaccines based upon immunity to the M2 extracellular domain (M2e) or conserved proteins, of which protective antibodies are not neutralizing. These antibodies are currently measured by ELISA which does not assess their biological protective activity and there is no antibody titer linked to protection for M2 antibodies [112, 143, 144]. The LAIV induces mucosal immunity and there are only poor correlates of protection associated with mucosal antibody and cellular immunity[145]. Additionally, evaluation of vaccines solely by HI titer does not consider CD8+ T cell-mediated protection. Attempting to correlate this immunity to protection is much more complex as it would have to take into account an individual's HLA phenotype, immunological status and influenza infection and vaccination history [114].

There are also inherent problems with the current influenza vaccines that impact their protective efficacy. Both inactivated and LAIV are not protective against antigenic drift viruses. Five different antigenic sites have been identified in the globular head of HA that determine antigenic properties and mutational changes in these sites can alter antigenicity or increase receptor binding affinity [146]. Suboptimal match or complete antigenic mismatch of the vaccine and circulating strains can affect the efficacy of these vaccines, leading to lower vaccine benefit. When the vaccine antigenically matches circulating strains, up to 90% of infections can be prevented however, antigen divergence can reduce protection to 30-40% of infections [147, 148]. In addition, these vaccines are not protective against antigenic shift viruses which are associated with pandemics such as the 2009 H1N1 or the 1918 "Spanish" flu. Finally, the amount of time required to create the vaccine is lengthy as most influenza vaccines require embryonated chicken eggs for production which necessitates early strain prediction [149]. At times, the predicted strain to circulate does not grow well in embryonated eggs such as in the 2003-2004 influenza season and vaccine shortage can occur if there are not enough embryos to grow virus or if the virus does not grow well [150]. This has been partially solved through the approval of cell-cultured

influenza vaccines [125] however, strains to be included in the vaccine are still predicted by the WHO. Further research needs to be conducted in developing more cross-protective, ‘universal’ vaccines that are not susceptible to antigenic mismatch and that would provide protection against pandemic strains.

### **Universal influenza vaccines**

While current seasonal influenza vaccines target the variable HA and NA glycoproteins for antibody-mediated protection, there are more highly conserved proteins or regions that could be targeted to induce cross-protective immune responses; two such candidates are M2 and HA2 stalk region.

#### **M2e**

The M2 ion channel protein has become a main target for vaccine induced immunity in universal vaccines. It is a highly conserved single pass type III transmembrane protein that forms homotetramers on the surface of the virion and is translated from a spliced variant of the mRNA coding for M1, thus restricting its sequence diversity [29, 151-153]. The transmembrane domain of M2 acts as a pH gated ion channel protein that is essential for release of the viral genome during viral entry by acidifying the virion interior after endocytosis [40, 154, 155]. The 54 amino acid cytoplasmic tail has also been shown to be important for incorporation of viral RNPs into infectious viral particles by interacting with M1 [59, 60, 64] whereas the 24 amino acid N-terminal extracellular domain does not have a known function. However, the M2e is highly conserved within a species but not across species [156]. While only about 16-20 molecules of M2 are incorporated into each virion, it is expressed abundantly at the apical plasma membrane of infected cells [153].

Despite being a weak immunogen [157], M2 is an attractive target for a universal vaccine based upon its high conservation. Naturally, antibodies against M2e are not induced, most likely

due to the immunodominant HA and NA but are protective *in vivo* from influenza challenge [144, 158]. *In vitro*, M2e antibodies reduce the size of influenza virus plaques, potentially due to blocking late stage replication, interactions with other viral proteins, or interfering with assembly and budding at the plasma membrane [144, 159]. Passive immunization with these antibodies in mice results in reduced viral lung titers and protection from mortality from IAV challenge but mice still become infected, exhibiting weight loss associated with morbidity [158, 160]. However, there is no known mechanism for M2e-mediated antibody protection. One study suggests that antibodies bound to M2e on the viral surface are recognized and the virion is removed by opsonophagocytosis by macrophages or NK cells [112, 161]. Several studies have shown that non-neutralizing anti-influenza humoral immunity is dependent on opsonophagocytosis of influenza virions by macrophages [100, 110, 162] with IgG2A isotypes being known to interact efficiently with complement and Fc receptors [110, 163-165]. Other studies have demonstrated that M2e based immunity is complement dependent or rely on an ADCC mechanism [112]. Depletion experiments using clodronate liposomes have shown the importance of DC and macrophages [143]. However, each study on the mechanism of M2e-antibody mediated protection uses a different form of M2e, either conjugated to an adjuvant, in a vector or as a pure protein, and thus the different ways of introducing the epitope may be affecting the type and quality of immune response that is generated and the mechanism of protection. Due to this, there is no true correlate of protection for these antibodies despite it being suggested that the induction of IgG1 and IgG2a antibodies provide the most protection [166-168].

Since the observation of the protective capacity of M2e antibodies, many investigators have attempted to provide cross-protective immunity by creating vaccines based upon M2. Slepushkin *et al* was the first to describe cross-protection by vaccinating mice with the full length M2 protein, purified from a baculovirus insect-cell expression system, in combination with incomplete Freund's adjuvant (IFA) [169]. Vaccinated mice demonstrated reduced morbidity,

mortality and viral titers in the lungs. Initially, the entire M2 protein was utilized for vaccination purposes but this immune response was further tailored to focus solely on the M2e. The first 24 amino acids of M2e are highly conserved across both IAV groups with 17 of these amino acids conserved at a rate of over 94% and 100% conservation occurs in the first nine amino acids [168, 170]. Along with this conservation, the M2e was described to be the most immunologically relevant portion of the protein [169, 171] and subsequent immunization of M2e conjugated to carrier proteins such as glutathione-S-transferase (GST) [171], human papilloma virus (HPV) L protein [172], keyhole limpet hemocyanin (KLH) [173], bacterial outer membrane complex [174] and other various carrier molecules provided homologous and heterologous protection [112, 167, 175-178]. It was also found that antibodies induced by vaccination with just M2e resulted in equivalent antibody titers to that of vaccination with full length M2 and the induction of IgG antibody was shown to be protective [167, 169, 171]. Several investigators have created a hepatitis B core (HBc) –M2e fusion protein which resulted in aggregates of highly immunogenic VLPs that induced long lasting protection against heterosubtypic lethal IAV infection after intraperitoneal (i.p.) or intranasal (i.n.) administration [156, 174, 179]. This protective efficacy was further enhanced by adding adjuvant leading to lower morbidity among mice.

To further enhance the immunogenicity of M2e, several strategies were utilized to present multiple copies of the M2e to the immune system. B cells require cross-linkage of immunoglobulin (Ig) receptors for activation of intracellular signaling events that lead to the maturation of antibody-secreting cells [180]. Four tandem copies of M2e was fused to flagellin, a TLR5 ligand [181] or to a GST fusion protein bearing multiple M2e copies [182]. The high M2e epitope density in a single recombinant protein molecule resulted in enhanced M2e-specific humoral responses, leading to higher survival rates in infected animals. Ernst *et al* used a liposomal based M2e vaccine where M2e was fused to a proprietary hydrophobic protein domain and was incorporated into the membrane of small unilamellar liposomes of 100nm in diameter

[178]. Three different M2e sequences were incorporated that represented potentially pandemic strains: an H1N1, H5N1 and H9N2. As the M2e is conserved within but not across species, this approach would be protective against potentially HPAIV viruses. This vaccine candidate stimulated the production of M2e specific IgG1 antibodies and mice were protected against various influenza strains [178].

While chemical and genetic conjugation of M2e have been used frequently to create an M2-based universal vaccine, there was some indication that these approaches may not present the epitope in its native tetrameric form. Antibodies induced by vaccination with M2e HBc conjugates were able to recognize M2 peptide but did not bind efficiently to M2 on virus particles [112] suggesting that despite M2e being the immunologically relevant portion of the protein, vaccination with whole WT M2 would be advantageous since it would likely retain its membrane-anchored native conformation. Vaccination with M2 VLPs, without the use of adjuvants, induced antibodies that recognized M2 on the cell surface and virus particles and provided protection from lethal challenges of different subtypes [183]. By mimicking the quaternary structure of M2e, oligomer specific antibodies were induced [184]. This was achieved by using M2e bound to a modified version of a leucine zipper from yeast transcription factor GCN4 (M2e-tGCN4). I.P. and i.n. vaccination resulted in high titer antibody that recognized M2e on the surface of virus infected cells or in an M2 expressing cell line, suggesting that M2e-tGCN4 chimeric proteins adopted the WT M2e structure. These immunized mice were 100% protected from a lethal dose of IAV [184]. A consensus M2e sequence has also been linked to the rotavirus fragment NSP4<sub>98-135</sub> that exists as a coiled-coil and is known to form tetramers in aqueous solution. This fusion vaccine induced an accelerated, augmented and more broadly protective antibody response when compared to M2e peptides and resulted in a significant decrease in lung viral titer [185]. It was subsequently found that M2-specific monoclonal antibodies that preferentially bound to M2 multimeric but not monomeric forms were

more protective, independent of NK-cell mediated effector functions [186], reinforcing the need for multimeric antigen presentation.

Many approaches have demonstrated the cross-protective efficacy of M2-based vaccines. However, while these vaccines protect against mortality, there is still a significant amount of morbidity observed as M2 antibodies are not neutralizing and do not prevent infection. DNA and viral vector vaccines have been used as a prime-boost regimen to improve not only humoral but also cellular immune responses [187, 188]. While the cellular immune response does not inhibit initial infection, it does aid in influenza clearance and replication suppression, working collaboratively with the humoral response [110, 112]. M2 contains T cell epitopes against which a cellular immune response can be elicited. Adenovirus (Ad) is a popular virus vector that has the potential to prime all aspects of the immune response to induce a far greater cell-mediated response without the use of adjuvants. Tompkins *et al* investigated the DNA/Ad vectored prime-boost approach by using a full-length consensus M2-DNA to prime the immune response followed by a boost with recombinant Ad (rAd) expressing M2 that was able to enhance cross-reactive antibody responses and induce a T cell response which conferred broad protection against lethal IAV challenge [173]. Subsequently, this approach was refined to include another conserved antigen, NP, in the rAd boost for a greater induction of heterosubtypic immunity [189, 190]. Results demonstrated that DNA prime-rAd boost with NP and M2 was protective against a lethal challenge with an H5N1 and induced a greater protective immune response than the LAIV which fails to induce detectable antibody to M2e [188]. More recently, rAd expressing M2e in a variable region of hexon, the most abundant capsid protein, and NP as a transgene demonstrated the induction of a robust M2e-specific antibody response that could be boosted upon a second dose and a CD8<sup>+</sup> T cell response against NP that reduced morbidity and was completely protective in outbred mice [189]. These studies show the immunogenicity and protective capacity not only of prime-boost approaches but of viral-vectored M2e vaccines.

Most M2e vaccines have been tested via intramuscular (i.m.) or i.p. injection but i.n. immunization has provided better protection despite the induction of lower levels of serum IgG specific for native M2 [166]. I.n. vaccination induces local airway associated immunity that involves IgA and M2e-specific B and T cells [191]. Immunity in the upper respiratory tract may operate more effectively against influenza infection as the virus replicates in respiratory epithelia. Unfortunately, there are no correlates of protection for mucosal-based immunity or M2e immunity and the mechanism of protection by vaccination of M2e is still not understood, thus necessitating further research into this area.

While vaccines based upon the induction of M2e-immunity demonstrate cross-protective properties, there are still many issues that need to be addressed before an M2 universal vaccine can be licensed. Natural infection or current influenza vaccines rarely induce M2 immunity and it is not known, if used on a global scale, if escape viruses would emerge [157]. No escape viruses were isolated after 11 passages of influenza viruses through M2e immunized immunocompetent mice, suggesting that the likelihood of emergence of a fit M2e escape virus is low but this still remains a possibility [192]. The level of protection conferred by current inactivated vaccines upon antigen match to circulating strains has not been achieved by M2 vaccines[193]. This is compounded by the lack of a clear mechanism and correlate of protection for M2 vaccines. Additionally, a major goal of vaccines is to neutralize the pathogen and completely prevent infection. However, this cannot be achieved by M2 universal vaccines as it is infection permissive and only serves to reduce disease symptoms and overall mortality [158, 160]; this is a realistic goal for a pandemic vaccine but would not be a suitable replacement for seasonal vaccines. Currently, it is highly unlikely that an M2 universal vaccine will be licensed as a ‘stand-alone’ vaccine given the current research but it would be suitable as an adjunct to current vaccines in order to provide increased cross-protection in case of an unanticipated emergence of a major drift variant or a new subtype. The addition of an M2e peptide enhanced cross-protection



in mice when immunized i.p. with an aluminum-adjuvanted split H3N2 virus [194]. Even more encouraging is that the supplementation of current inactivated influenza vaccines with M2 VLPs completely prevented disease symptoms with no observed weight loss and this combination conferred cross-protection against lethal challenge with heterologous virus [143]. Thus, M2e vaccines still remain a viable option to confer greater cross-protection on the way to creating a ‘universal’ vaccine.

### **HA stalk**

Another potential target for broadly cross-protective universal influenza vaccines is the stalk region of the HA protein. There are two phylogenetic groups of HA: group 1 and group 2 with notable members of group 1 including H1, H5 and H9 while group 2 includes H3 and H7 [106]. HA is a homotrimeric glycoprotein that consists of a disulfide-linked globular head of HA1, containing the receptor-binding pocket, and a stem composed of HA1 and HA2 which encapsidates the fusion peptide (Figure 1B) [195]. Protease-mediated cleavage of HA0 into HA1 and HA2 is required for fusion and productive replication [38, 69, 70]. This cleavage forms an extended, highly exposed loop structure on the surface. Current seasonal influenza vaccines induce neutralizing antibodies to the antigenically variable globular head of the HA protein and prevent viral attachment to the host cell [146]. However the HA protein does possess conserved structures and sequences in the HA2 segment that is anchored to the viral membrane called the stalk.

HA2 represents the carboxy-terminus of HA and forms the stalk structure [196]. The fusion peptide is located at the amino terminus of the HA2 protein and is considered to be one of the most highly conserved regions of all influenza HA proteins [197, 198]. This region is invariant in influenza A viruses and only differs by one or two conservative amino acid replacements in influenza B [199]. Overall, the HA2 subunit has 85% sequence homology among

different subtypes and 95% homology within strains of the same subtype [200]. Antibodies targeting the stalk region are cross-reactive within a group but not across HA groups due to the high sequence conservation within each group. The invariance of this domain is likely due to functional constraints as a series of conformational rearrangements of HA are required to mediate fusion [39]. At a neutral pH, the amino-terminus of the fusion peptide is inserted into the interspace of the HA trimer whereas at an acidic pH, this terminus is exposed and inserted into the endosome membrane to mediate fusion and allow for the RNP complex to be released into the cytoplasm [201-203]. The fusion peptide is accessible to antibody on the uncleaved HA precursor that is exposed on the plasma membrane of infected host cells as it is located in an exposed loop. However, during natural infection or vaccination with seasonal influenza vaccines, the immune response against the fusion peptide or HA2 stalk are very weak, most likely due to the immunodominance and bulk of the globular HA1 head domain, making the stalk epitope less accessible to antibody binding [197, 204, 205].

The ability to elicit broadly protective antibodies against HA2 began when the high sequence homology between HA2 as compared to HA1 subunits was noticed [204]. To determine if HA2 possessed antigenic properties, mature virus particles were treated with acid followed by dithiothreitol (DTT) or trypsin in order to alter the HA conformation and remove HA1, resulting in HA2 being displayed by both procedures. These particles were injected into rabbits and three weeks post injection, antiserum was collected to evaluate the humoral response. Surprisingly, the rabbit antisera detected HA from both H1 and H3 subtypes, demonstrating cross-reactivity between groups [204]. It is unlikely, however, that antibodies of this nature would be induced by the immune response as the reduction in pH prior to cleavage stimulated a conformational change in HA2 that would not naturally occur and are not physiologically relevant.

However, more recent studies have investigated different methods to induce antibodies against HA2. The first HA2 cross-reactive antibody, C179, was identified through the use of a hybridoma library made from mice immunized with an H2 strain and recognized group 1 subtypes [206]. This single monoclonal antibody, upon further investigation, was broadly protective but did not prevent attachment of HA in an HI assay. Only HA1 antibodies that prevent attachment are detected by HI assays and mapping of the binding region of C179 revealed that this antibody bound to a conformational epitope on HA1 and HA2, with most contacts made in the stalk region [206, 207]. It was later discovered that C179 inhibited syncytia formation that is characteristic of normal HA fusion, suggesting that antibodies targeting this region are protective by preventing fusion of the virus to the endosomal membrane. Mechanistically, these antibodies could insert its heavy chain into the conserved pocket of the stem region or prevent HA conformational changes necessary to reveal the fusion peptide [105, 106, 206]. Passive and active immunization of HA2 stalk antibodies led to neutralization of the virus, greatly decreasing morbidity and mortality [206].

Several methods were undertaken to increase the frequency of inducing broadly protective HA antibodies. The immunogenicity of HA2 was increased by removing the immunogenic globular head of HA1 [197]. These ‘headless’ HA trimers still form the conserved stalk domain so HA2 epitopes are more accessible to B cells than in native HA, leading to the discovery of more broadly cross-reactive antibodies [197, 205]. As C179 recognized an epitope that spanned HA1 and HA2, mice vaccinated with a peptide spanning the HA1-HA2 connecting region exhibited milder illness and fewer deaths upon a lethal viral challenge [208-210]. Furthermore, immunization with chemically modified fusion peptides conjugated to KLH induced antibodies that reacted to different subtypes of HA by specifically recognizing the fusion peptide sequence [183].

The identification of further cross-reactive HA2-based monoclonal antibodies has been achieved through phage display and combinatorial libraries. A highly cross-reactive antibody, CR6261, was isolated from a healthy, vaccinated individual through the use of phage display selection on recombinant H5 HA [106]. This IgG1 antibody neutralized multiple influenza subtypes from group 1 HA and prophylactically and therapeutically protected mice 5 days post infection with a lethal challenge of H1N1 and H5N1 viruses. Crystal structure analysis of the CR6261 antibody in complex with HA's from the 1918 H1N1 pandemic virus and an HPAI H5N1 virus revealed that it primarily bound to the HA2 central alpha helix (HA2 A; Figure 1B) with some contacts in the HA1 stem region [106]. Importantly, the HA2 A region is required for proper trimerization and pH induced unfolding [211]. The identification of this new conserved epitope helps to accelerate the design and implementation of improved therapies and/or vaccines based upon HA2-mediated immunity .

A similar approach was used by Sui *et al* to identify F10, a monoclonal antibody that recognizes a conformational epitope comprised of HA1, HA2 A and HA2 [105]. Prophylactic and therapeutic administration of this antibody protected mice from lethal doses of H5N1 and H1N1 viruses and neutralized virus by inhibiting cell fusion. Similar to CR6261, there was high sequence conservation within the binding region of F10 which enabled this antibody to neutralize across group 1 strains. Crystal structure analysis demonstrated that F10 bound preferentially to two important tertiary structures that are conserved across all of these subtypes. Multiple F10-HA contacts were made in the HA2 A region that CR6261 also recognized, with the center of the binding epitope recognizing four hydrophobic amino acids that comprise a portion of the fusion peptide and is conserved across all IAV, including group 2 viruses [105]. However, the inability of F10 to recognize group 2 viruses is due to the glycosylation of residue 39 and 40 of HA2 in group 2 viruses, along with the orientation of tryptophan at position 21, which prevents epitope recognition and subsequent heterosubtypic protection [105, 212]. Importantly, nine out of ten

neutralizing antibodies discovered by this study employed the same heavy chain gene usage as CR6261, *VHI-69* [105, 212], suggesting that this conserved gene usage can be induced in response to the correct conformational HA2 antigen.

The first broadly reactive antibody against H3 viruses, 12D1, was isolated from mice that had been sequentially immunized with DNA coding for HA from three antigenically distinct H3 viruses [213]. Of the group 2 viruses, H3 is currently the only human IAV thus, although 12D1 was unable to neutralize other group 2 subtypes, it could still protect from seasonal group 2 influenza strains. Similar to CR6261 and F10, 12D1 prevented viral fusion however, unlike the group 1 broadly reactive antibodies that recognize a conformational epitope, 12D1 binds to a continuous region of HA2, the long alpha helix (LAH; Figure 1B). This suggests that unlike the non-contiguous epitopes of the broadly reactive group 1 mAb, the LAH could serve as an effective immunogen. A synthetic peptide containing the LAH of H3 was coupled to KLH and elicited protective antibodies against the stalk of the HA protein [213]. While the induced antibodies were most protective against H3 viruses, they demonstrated slight protection from H5N1 and H1N1 viruses, with less morbidity and a delay in time to death. Importantly, multiple passages of H3 viruses in the presence of 12D1 did not select for escape mutants, most likely due to the secondary helix structure required for stabilization of the HA trimer and this suggests that a vaccine based upon immunity to LAH would not lead to significant antigenic drift [213].

Monoclonal antibody, F16, that neutralizes both group 1 and group 2 viruses was discovered by Corti *et al* through a sensitive, high throughput screening method, utilizing IL-6 supplemented PBMCs from human volunteers [214]. F16 bound to both recombinant and purified HA from all group 1 and group 2 viruses tested *in vitro*. Furthermore, this monoclonal antibody prevented lethality and weight loss in mice challenged with H1, H3 and H5 viruses, highlighting its ability to overcome structural differences that have previously limited heterosubtypic antibodies from being cross-reactive to all IAV subtypes. Crystal structure

analysis of F16 in complex with HA demonstrated that unlike previously described group 1 monoclonal antibodies that interact primarily through the heavy chain [105, 212], F16 uses both heavy and light chains to make contacts with HA1 and HA2, likely a result of multiple somatic mutations. This allows for added flexibility due to different VH gene usage to overcome steric hindrance at Trp21 and glycosylation at residue 38 [214].

While the isolation and identification of F16 from humans is encouraging for the future of universal vaccines, this antibody is extremely rare and was identified in only 4 out of 104,000 plasma cells screened [214]. Even heterosubtypic antibodies like CR6261, F10 and 12D1 are uncommon, with F10 being identified out of a 27 billion human-antibody phage display library [105, 212, 213]. There are several potential reasons for the rarity of these broadly neutralizing HA antibodies. The regions of HA2 that group 1 and group 2 heterosubtypic antibodies recognize are proximal to the viral membrane and are physically blocked by the globular head domain of HA1 [197]. Additionally, the globular head of HA1 is very immunodominant, with the majority of monoclonal antibodies identified targeting conformational epitopes that are likely presented often by MHC molecules. Antibody production is dependent upon B-cell peptide recognition within the context of the MHC and if the conserved regions of the HA stalk are not presented, stalk antibodies would not be created [215]. Finally, the F16 antibody required multiple somatic mutations to increase the binding interface necessary to recognize structurally distinct group 1 and group 2 HA stalk regions [214]. The prevalence of mutations such as these are not known but are probably uncommon, based upon the rarity of the F16 antibody. Thus the infrequency of these broadly protective antibodies cautions the use of HA2 as a vaccine antigen.

The immunization of individuals with a conserved epitope recognized by broadly protective antibodies does not guarantee the natural induction of a heterosubtypic immune response. Since several broadly neutralizing antibodies such as CR6261, 12D1 and F10 primarily make contacts with the HA2A, it was investigated whether the HA2A region itself was sufficient

to elicit broadly neutralizing antibodies. HA2A was displayed on the capsid of Flock House Virus VLPs in a small helix-turn-helix scaffold to maintain secondary structure of the peptide [176]. While this VLP did display HA2A in the correct conformation, few mice produced antibodies against this region and they were not protective. This suggests that the HA2A alone is not sufficient as an antigen to induce broadly neutralizing antibodies which is a common difficulty in vaccine development. To overcome dependence on the immune system to develop a protective response against influenza, protective antibodies can be produced *in vivo* to bypass the uncertainty of immune induction by conventional antigens. This system, termed vectored immunoprophylaxis (VIP) was developed in David Baltimore's laboratory and used an adeno-associated virus serotype 8 (AAV8) vector to express human IgG monoclonal antibodies CR6261 and F10 that was encoded as a transgene [216, 217]. Upon a single intramuscular injection of the AAV8 vector, these monoclonal antibodies were expressed to high levels *in vivo* by one week post transduction and protected mice from challenge by group 1 influenza viruses. While it has already been known that passive administration of antibodies is protective, this system allows for the expression of these antibodies *in vivo* rather than injection of *in vitro* cultured monoclonal antibodies. Additionally, it was able to protect both young mice and immunocompromised elderly mice, two highly susceptible populations to influenza complications that either do not respond as well or are not suggested to receive current seasonal vaccines [217]. This demonstrates a prophylactic approach that could be utilized in combination with some of the well characterized broadly protective monoclonal antibodies for a universal vaccine approach.

### **Cross-reactive cytotoxic T lymphocytes**

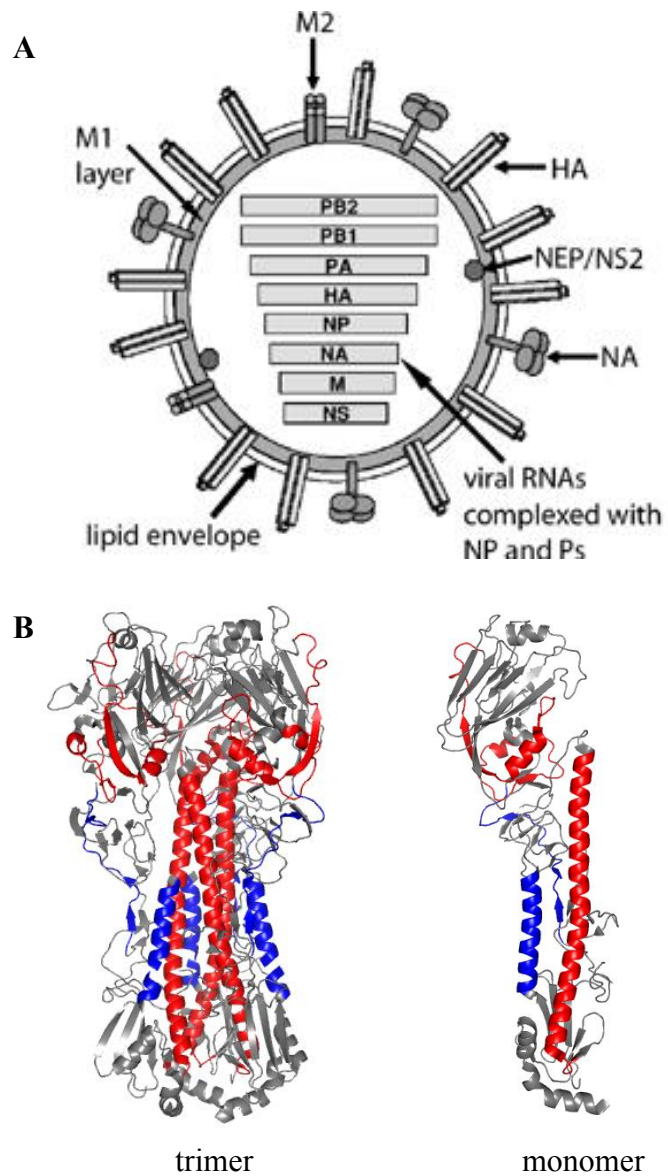
Seasonal influenza vaccines and most universal vaccine candidates rely upon the induction of protective antibodies however cell-mediated immunity does play a role in the control of influenza virus infections. Cytotoxic T lymphocytes (CTL) can eliminate virus infected cells by recognition of viral antigens presented on MHC I to contribute to the clearance of virus from

infected tissue [218, 219]. Depletion of T cells in IAV infected mice have led to higher viral titers in the lung, increased mortality and more severe disease [219]. While CTL can be subtype specific, of importance to the development of a universal vaccine, they can also be broadly cross-reactive, depending upon the antigen. Early studies in influenza infected mice demonstrated that a majority of influenza-specific CTLs were cross-reactive across subtypes and this has also been described in naturally infected humans [115, 220]. This high crossreactivity can be explained by the antigenically conserved internal targets of CTL, mainly targeting NP, M1 and NS1 [114, 116, 221, 222].

While heterosubtypic immunity can be generated against conserved CTL epitopes, it is not a promising ‘universal’ influenza vaccine approach for multiple reasons. First, there is immunodominance of CTL epitopes where only a small fraction of peptides created by proteasomes are presented by MHC I molecules and recognized by CTLs [215, 223]. This could be due to the HLA haplotype and its binding affinity to individual epitopes, repertoire of T-cell receptors, processing and presentation of viral peptides and interaction of CTL with APC [215, 223], making it difficult to predict what conserved epitopes will be protective in a majority of the population. Additionally, the efficiency of epitope processing is not the same for all epitopes and this has been shown to affect immunogenicity. Most vaccines are first assessed for immunogenicity in a mouse model however, it is difficult to assess T cell-based vaccines as the most frequently used models are inbred mice: B57BL6 (H-2<sup>b</sup>) and BALB/c (H-2<sup>d</sup>) [224] with defined HLA genes. While certain T cell epitopes may be immunodominant in these mice, not all epitopes are equally immunogenic thus it is hard to translate these epitopes into humans where the HLA genes are highly polymorphic. While there are epitopes, such as M1<sub>58-66</sub>, that are recognized by multiple HLA genes, only 80-90% of the population would be protected [225]. Ultimately, T cell responses are dependent upon an individual’s genetics, making mass vaccination of a CTL-based influenza vaccine unrealistic. Furthermore, there is no known



correlate of T-cell mediated protection, making the assessment of vaccines even more difficult. Thus, the feasibility of designing an effective universal CTL epitope vaccine in humans is questionable without further knowledge and investigation into broadly protective epitopes across HLA haplotypes and correlates of protection.



**Figure 1: Influenza A virus structure**

(A) Schematic diagram of a spherical influenza A virus particle [46]. The hemagglutinin (HA), neuraminidase (NA) and M2 protein are inserted into the lipid envelope derived from the host cells. Both M2 and NA are tetramers while HA exists as a trimer. M1 is associated with the

lipid envelope on the interior of the virus particle. The nuclear export protein (NEP)/NS2 protein is also incorporated into the virion. The viral RNA exists as 8 segments coated with nucleoprotein (NP) and are bound to the polymerase [46]. (B) Ribbon diagram of HA trimer (left) and monomer (right). HA2 alpha (HA2A) helix is labeled in blue (residues 38-58) and the long alpha helix (LAH) is labeled in red (residues 76-130). Produced using program PYMOL and PDB 3GBN [106].

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## **Chapter Two:**

**The highly conserved influenza A virus M2 extracellular domain is not  
important for virus replication**

## **Abstract**

The influenza A virus M2 protein has essential roles in both virus entry and assembly of infectious virus particles. These functions have been mapped to the transmembrane and cytoplasmic domains, respectively. However, there is currently no known function of the extracellular domain of the M2 protein (M2e). The M2e is highly conserved suggesting that it is important for M2 function. To investigate the role of the M2e, triple alanine substitution mutants spanning the entire 24 amino acid domain were made and expressed in MDCK stable cell lines. Mutant M2e function was assessed by transcomplementation assays of M2-null viruses. Neither directed alanine mutagenesis nor the deletion of the first nine amino acids of the M2e attenuated replication. This suggests that the high conservation may be due to a functional role of the differentially spliced M1 protein or lack of immune pressure causing sequence conservation.

## Introduction

Influenza A virus (IAV) is a member of the *Orthomyxoviridae* family whose genome consists of 8 negative-sense RNA segments that code for 10 or 11 proteins [1]. Segment 7 encodes the highly conserved 97 amino acid M2 protein as well as M1 by differential splicing [2]; therefore, M2 and M1 share the first 8 amino acids. M2 is a disulfide-linked tetrameric type III integral membrane protein with an extracellular amino terminus and an intracellular carboxy terminus. In the viral life cycle, M2 is required for virus entry, where it acts as a pH-gated, proton-selective ion channel and translocates ions into the virion interior [3]. This mediates the release of viral nucleoprotein complexes (vRNPs) from M1, allowing for vRNP transport to the nucleus for RNA transcription and replication [4-6]. The ion channel, in some influenza A species, also stabilizes the conformation of the HA protein in the trans-Golgi network [7]. Sequences in the M2 cytoplasmic tail are essential for the formation of infectious virus particles at the plasma membrane by mediating incorporation of NP and vRNPs into budding particles [8-11].

The roles of the ion channel transmembrane domain and cytoplasmic domain of M2 in the viral life cycle have been well defined [3, 8-10, 12]. The 24 amino acid M2 extracellular (M2e) domain is highly conserved across all influenza A species which suggests that it is important for M2 function. Despite this hypothesis, the role of this region in the viral life cycle is largely unknown. One study suggests that the M2e is important for incorporation of M2 into virus particles [13] but there are no known interactions between M2e and the ectodomains of other viral glycoproteins.

To systematically investigate the role of the M2e domain in virus replication, triple alanine substitution mutations were made across the entire region and expressed in MDCK stable cell lines. M2 function was assessed through transcomplementation assays with M2-null viruses.



All mutants replicated successfully as demonstrated by growth curves, suggesting that the M2e can tolerate numerous mutations without any apparent adverse affect on virus replication.

Similarly, mutation or deletion of the first eight completely conserved amino acids led to no attenuation of the virus. Therefore, despite the high conservation of the ectodomain, this region is not critical for the function of M2, as assessed by transcomplementation, in viral replication *in vitro*.

## Materials and methods

### *Plasmids and mutagenesis.*

The plasmid pCAGGS [14] encoding the cDNA for the full length M<sub>2</sub> protein from A/WSN/33 (pC WSN M2), has been described previously [10]. M2 ectodomain mutations were introduced into pC WSN M2 by 4 primer overlap extension PCR [15, 16]. All inserts into plasmids were confirmed by sequencing.

### *Cells*

Madin-Darby canine kidney (MDCK) cells and human embryonal kidney (293T) cells were cultured in Dulbecco's modified eagle medium (DMEM; Sigma) containing 10% fetal bovine serum (FBS; Atlanta Biologicals), 100 U/mL penicillin (Invitrogen), 100 µg/mL streptomycin (Invitrogen), 1mM sodium pyruvate (Sigma) and 2mM Glutamax (Invitrogen) at 37°C and 5% CO<sub>2</sub>.

All cell lines that stably express M2 WSN N31S [16] or M2 mutants were cultured in identical media as wild-type MDCK cells and were supplemented with puromycin (7.5 µg/mL; Sigma) and amantadine HCl (5 µM; Sigma). The N31S mutation allowed for amantadine sensitivity to the M2 protein of A/WSN/33 virus and was added to culture medium to prevent toxicity from an active ion channel [3, 17]. Stable cell lines expressing mutant M2 ectodomain were generated by cotransfecting MDCK cells with plasmids expressing a puromycin resistance gene (pBABE) [18] and a pCAGGs M2 expression vector in six-well plates. Two days post transfection, the cells were trypsinized, subjected to puromycin selection and cloned by limiting dilution in 96 well plates. Cells were screened for M2 expression by indirect immunofluorescence of live cells for cell surface-expressed M2 and positive clones were detected

with a fluorescent plate reader. Cell lines were screened by flow cytometry for homogenous M2 expression by flow cytometry.

#### *Flow cytometry*

Cells were removed from the tissue culture plate by trypsinization. The cells were stained for M2 surface expression using monoclonal antibodies 14C2 (1:500 dilution) [19] or TCN-032 (1:50 dilution; Theraclone Sciences) [20] followed by Alexa Fluor 647-conjugated secondary antibodies goat anti-mouse immunoglobulin (IgG) or goat anti-human IgG (all at a 1:500 dilution; Invitrogen). C terminal flag-tagged M2 cell lines were fixed by 2% paraformaldehyde (Sigma) followed by permeabilization with 0.2% TritonX (Sigma). Prior to analysis, all cells were fixed with 2% paraformaldehyde at room temperature (RT) for 15 min. The cells were analyzed by flow cytometry (Becton Dickinson FACS Calibur) by using FlowJo software.

#### *Viruses*

Viruses used in this study were functionally M2-null viruses, rUdorn M2Stop and rWSN M2Stop [10], which are recombinant viruses of A/Udorn/72 [H3N2] and A/WSN/33 [H1N1] [3, 21] that encode a truncated M2 protein as a result of codons 25 and 26 being altered to stop codons [9]. These viruses were propagated on MDCK cells stably expressing WSN M2 N31S in DMEM containing 4 µg/mL of *N*-acetyltrypsin (Sigma), 100 U/mL of penicillin, 100 µg/mL of streptomycin and 0.3% bovine serum albumin (BSA; Sigma).

#### *Virus infections*

Low-multiplicity growth curves were performed at a multiplicity of infection (MOI) of 0.001 50% tissue culture infectious dose (TCID<sub>50</sub>) per cell. For complementation assays, MDCK cells expressing mutant or WSN M2 N31S were infected with M2 stop viruses. Confluent cells grown in 6-well plates were washed twice with phosphate-buffered saline with calcium and magnesium

(PBS+; Invitrogen) to remove FBS. Cells were infected with indicated viruses in 500  $\mu$ L infection medium (DMEM supplemented with 0.5% BSA, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 1 mM sodium pyruvate, 2 mM Glutamax and 4  $\mu$ g/mL *N*-acetyl trypsin [NAT; Sigma]) at RT with rocking for 1 hr. Cells were then washed twice with PBS+ and incubated with 1 mL infection medium at 37°C. At indicated time points, the media was removed, stored at -80°C and replaced with fresh infection medium. The amount of infectious virus was determined by TCID<sub>50</sub> assay on MDCK cells expressing M2 WSN N31S.

#### *TCID<sub>50</sub> assay*

MDCK cells expressing WSN M2 N31S were plated in 96-well plates. Upon confluency, cells were washed twice with PBS+, infected with 100  $\mu$ L of ten-fold serially diluted virus in replicates of 4 and incubated for 4 days at 37°C. Cells were fixed by adding 50  $\mu$ L of 4% formaldehyde (Fisher) in PBS, stained with Naphthol Blue Black solution, and visually scored for cytopathic effect. The TCID<sub>50</sub> was calculated by the method of Reed and Muench [22].

#### *Microscopy*

293T cells grown to 10-15% confluence on tissue culture-treated glass coverslips (Fisher Scientific) in 6 well plates were transfected using 1.5  $\mu$ g of pCAGG M2 mutant plasmid DNA and 2.5 times LTI (Mirus Bio). At 24 hours post transfection, cells were incubated on ice, washed twice with PBS and blocked for 30 min on ice with 3% normal donkey serum (Sigma) and 0.5% BSA in PBS. All further antibody dilutions were made in blocking media. Lectin staining was performed for 1 hour on ice in blocking solution using wheat germ agglutinin Alexafluor 555 (WGA; 1:500 dilution; Invitrogen). Cells were washed three times with PBS, fixed for 15 minutes with 2% paraformaldehyde at RT followed by permeabilization with 0.2% TritonX for 20 min at RT. Coverslips were removed from the 6 well plate and were washed 15 times by immersion in 0.2% Tween in PBS and blocked for 45 minutes in a humidifying

chamber. The cells were stained with anti-Flag antibody 14C2 (1:1000; Stratagene) for 1 hour. Coverslips were washed 15 times by immersion in 0.2% Tween in PBS and incubated in goat anti-mouse Alexafluor 488 (1:500; Invitrogen) secondary and 4', 6-diamidino-2-phenylindole (DAPI; 1:1000; Roche Molecular Biochemical) for 1 hour. Cells were washed fifteen times in 0.2% Tween in PBS and five times with deionized water before being mounted with ProLong Gold antifade reagent (Invitrogen).

Samples were imaged on a Nikon Eclipse 90i epifluorescence microscope. Ten non-overlapping pictures were taken of each sample using a 40x objective. For each image, colocalization of anti-flag and WGA staining was examined to determine if M2 was present at the cell surface.

#### *Sequence alignments*

All influenza A M2 protein sequences were obtained from the NCBI Influenza Virus Sequence Database [23]. Sequences, excluding 2009 pandemic H1N1, were aligned using ClustalW 2.0.10 [24]. The percent conservation of all influenza A virus M2 sequences encoding the human consensus M2e residue at each amino acid position was determined using WebLogo 3 [25].

#### *Statistical analysis*

Growth curves were analyzed using mixed analyses of variance (ANOVAs) with time and virus as independent variables for transcomplementation assays. All analyses were done using Prism 4.0 (GraphPad Software Inc).

## Results

### *Expression of mutant M2 proteins in stable cell lines*

Influenza A viruses encode an M2 protein with a highly conserved ectodomain (Figure 1) however the function of this region is unknown. To understand the biological function of this conserved region, triple alanine substitution mutations were made spanning the entire 24 amino acid ectodomain (Table 1). Mutations were not made at the starting methionine or cysteine residues 17 and 19 that are involved in disulfide linkages in order to maintain oligomeric assembly and prevent destabilization of the M2 tetramer [26].

MDCK cells were cotransfected with pBABE, to confer selective puromycin resistance, and a pCAGGs M2 expression vector using lipofectamine. Two days post transfection, cells were subjected to puromycin selection and were diluted such that a single cell occupied one well of a 96 well plate to obtain clonal, homogeneous colonies. Stably transfected MDCK cell lines were selected that constitutively expressed the wild type (WT) or M2e proteins. To determine if the mutant M2e proteins were expressed at the cell surface, two different monoclonal antibodies, 14C2 and TCN-031, were used that recognize the extracellular domain (Figure 1). Flow cytometry was performed on live cells and the total amount of surface mutant M2e expressed in the stable cell lines was comparable to a control cell line expressing WT M2 protein (WSN M2 N31S) (Figure 2A and B). This level of expression is above the level that is required to complement M2-null viruses [9]. Detection of the mutant M2e protein by flow cytometry suggests that the mutations did not affect the M2e native secondary structure since the epitope recognized by the TCN-032 antibody, used to recognize these constructs, is conformational [20] (Figure 2B).

Although 14C2 and TCN-032 recognize distinct regions of M2e, 5-7 Ala, 8-10 Ala and 14-16 Ala M2e constructs were not reliably detected (Figure 1). Inconsistent results with attempting to

detect the 5-7 Ala construct were most likely a result of partial antibody binding due to the mutation at amino acid 6. In order to detect M2 levels in 5-7 Ala, 8-10 Ala and 14-16 Ala M2e cell lines, these constructs were tagged with a flag epitope, DYKDDDDK, at the cytoplasmic tail (Table 1). The addition of an antibody epitope tag to the M2 protein cytoplasmic tail does not affect its expression or ion channel activity [27] and previous data (unpublished) has demonstrated that a flag epitope tag does not affect the ability of M2 to complement an M2-null virus. Because the epitope tag was added to the M2 cytoplasmic tail, it is impossible to determine the levels of cell surface expression however total expression could be detected. Flow cytometry was performed on fixed, permeabilized cells and the total amount of mutant M2e expressed in these stable cell lines was comparable to a control cell line expressing a flag-tagged WT M2 (WSN M2 N31S Flag) (Figure 2C).

#### *M2e proteins complement M2-null viruses*

To determine the effects of M2e mutations on M2 function, a complementation assay was utilized [9]. Stable cell lines expressing M2e mutations were infected with two strains of M2-null viruses, rWSN M2Stop, an H1N1 virus, (Figure 3A and C) and rUdorn M2Stop, an H3N2 virus (Figure 3B and D) in a modified TCID<sub>50</sub> assay measuring endpoint titers. Although all M2e mutations were made in the rWSN background, rUdorn M2Stop was used to determine if any of the mutated residues contributed to strain specific differences in virus complementation as assessed by virus replication. Neither rWSN M2Stop or rUdorn M2Stop were able to produce a high endpoint titer on MDCK cells that did not express M2, compared to WT M2 N31S cells (Fig 3). All mutant M2e cell lines were capable of complementing both rWSN M2Stop and rUdorn M2Stop to equivalent titers as WT M2 N31S cells indicating that all mutated M2 proteins were capable of supporting several rounds of virus replication (Fig 3).

In order to determine if M2e mutations had an effect on virus growth kinetics, these cell lines were infected at a low-MOI (MOI of 0.001) with rWSN M2Stop (Figure 4A, C, E and G) or rUdorn M2Stop (Fig 4B, D, F, and H) to allow for multiple rounds of virus replication. MDCK cells not expressing M2 did not support replication of either M2-null viruses (Figure 4). Although there were minor differences in replication kinetics, all mutant M2-expressing cell lines were capable of supporting replication of rWSN M2Stop and rUdorn M2Stop and reached equivalent titers at 48hpi as compared to WT WSN M2 N31S (Figure 4). The viral growth kinetics of viruses on mutant M2e expressing cells indicated that the conservation of these amino acids is not critical for M2 function as assessed by viral replication.

*Mutation of the conserved first nine amino acids of the M2e does not affect M2 function*

The entire M2e is more than 50% conserved, however the first 9 amino acids are 100% conserved across all influenza A viruses (Figure 1) suggesting that it may play an important role in M2 function. To determine if the entire region is essential for M2, two different constructs were created, one in which the 9 amino acids were mutated to alanines (2-9 Ala) and the other where these residues were deleted (2-9 Del) (Table 1). Both constructs were flag-tagged at the M2 cytoplasmic tail for detection.

The M2 protein is a type III integral membrane protein [28] and it is possible that the mutation or deletion of 9 amino acids from the M2e might impact the ability of M2 to target to the plasma membrane. This would inhibit the M2 protein from acting as an ion channel protein and complementation would not occur. Prior to generating stable cell lines, confocal microscopy was used on 293T cells transfected with plasmids expressing 2-9 Ala Flag and 2-9 Del Flag. These cells were stained with WGA, as a marker for the plasma membrane, and anti-Flag. Significant co-localization was seen between the WGA and M2e flag-tagged mutants (Figure 5A). These mutants exhibited similar cellular staining as 293Ts transfected with WT M2 N31S



Flag, suggesting that 2-9 Ala Flag and 2-9 Del Flag M2 proteins were located at the plasma membrane.

Stable cell lines expressing 2-9 Ala Flag and 2-9 Del Flag were created. Flow cytometry analysis showed M2 expression above the threshold needed to complement M2-null viruses [9] (Figure 5B). These cell lines were capable of supporting high titer virus replication of both rWSN M2Stop and rUdorn M2Stop (Figure 6A and B) and there was no statistically significant difference in the replication of the M2-null viruses on 2-9 Ala Flag and 2-9 Del Flag cell lines as compared to the WT M2 N31S Flag cell line (Figure 6 C-F). This suggests that the reason for the complete conservation of these amino acids is not due to a role that they play in M2 function in this assay.

## Discussion

The 24 amino acid sequence of the M2e is highly conserved but with no known function in the viral life cycle (Figure 1). We created stable MDCK cell lines expressing triple alanine substitution mutants spanning the 24 amino acid region (Table 1, Figure 2). All of the M2e mutants were capable of complementing a homologous and heterologous strain of IAV that both lack functional M2 (Figure 3 and 4). This suggests that the M2e is not critical for M2 function in the viral life cycle. To further investigate the conservation of the first nine amino acids that are 100% conserved, we made two flag-tagged stable cell lines where those residues were all mutated to alanines or were deleted. Using immunofluorescence, both constructs exhibited staining patterns similar to WT M2 N31S in transfected 293T cells and appeared to colocalize with WGA suggesting that the mutation did not affect the ability of M2 to traffic to the plasma membrane (Figure 5A). Stable cell lines expressing the 2-9 Ala Flag and 2-9 Del Flag M2e mutants (Figure 5B) were capable of complementing both rWSN M2Stop and rUdorn M2Stop and reached similar peak titers as WT M2 N31S (Figure 6). Even drastic mutations to a completely conserved region had no apparent affect on virus replication *in vitro* and further demonstrated that the first nine amino acids are not essential for M2 function in this assay.

The ability of M2 to tolerate mutations in a conserved region suggests that there is another explanation for the conservation of the M2e. Differential splicing of the M segment results in M1 and M2 sharing its first 8 amino acids [2, 29]. Thus any mutations to M2 in the virus would also affect M1 which could result in deleterious mutations. However, by complementing M2 null viruses in trans, we were able to circumvent mutating M1 and solely look at the function of these residues in M2. Since there was no defect in growth kinetics when M2 was mutated, this region may be completely conserved because of a function in M1. It would be interesting to take this same approach and determine if cell lines expressing M1 mutations in this region would be able to complement functionally null M1 viruses. Another approach would be to make the mutations

of the first 8 amino acids in the virus itself. This has not been done because it would not be possible to determine if a mutant phenotype was due to the mutation in M1 or in M2. Since this study has demonstrated that mutating the first 8 amino acids in M2 has no effect on viral replication, any phenotype seen in the virus would be due to the mutations in M1.

It is also possible that the mutations made in the M2e could have an effect on viral replication and virulence *in vivo*. Several mutations in the M2 protein have been described that exhibited little to no phenotype when investigated *in vitro* but demonstrated decreased virulence *in vivo* [16, 30, 31]. It would be interesting to make the M2e mutations in viable virus in order to assess the effect on replication in an animal model.

Due to its high conservation, the M2e is an attractive universal IAV vaccine target. Although monoclonal antibodies raised against M2e are not neutralizing, they are capable of protecting animals from morbidity and mortality [19, 32]. However, natural infection of humans results in no significant M2 antibody responses [11, 22, 33]. It is possible that this region has maintained its conservation because of the lack of immune pressure that would result in escape mutants. This brings up an interesting dilemma of whether a universal vaccine based on antibodies to the M2e would be effective or result in the selection of M2e escape mutants. Virus grown in the presence of M2e monoclonal antibodies in a mouse model resulted in escape mutants in 65% of the animals [34]. However, the diversity of these escape mutants was extremely restricted and only resulted in two mutations: P10H and P10L. There still remains the possibility that a polyclonal M2 antibody presence in the population would result in more diverse M2 escape mutant viruses.

Our results contrast with a study by Park *et al.*, who demonstrated a role for the M2e in incorporation of M2 into virus particles [13]. In that study, chimeric mutants of M2 and Sendai virus F protein were made where the corresponding extracellular (499 amino acids), transmembrane (24 amino acids) and cytoplasmic (42 amino acids) domains were swapped.

Virions were then tested to see if any Sendai virus F protein in the chimeras was incorporated into influenza virions. Of the chimeras, only three expressed at the cell surface and of these three, only one was incorporated into virus particles. This incorporated mutant expressed the extracellular domain from M2 and the remainder from Sendai virus F protein. The other two mutants that expressed at the cell surface contained the extracellular domain of the F protein and were not incorporated, suggesting a role for the ectodomain in M2 incorporation. We took a more thorough approach to the role of the M2e and made stable cell lines expressing scanning alanine mutations of the M2e, all of which were capable of complementing M2 null viruses. Since low MOI growth curves were used to characterize these cell lines, this data not only suggests that the M2e does not affect M2 function but that it also does not affect M2 incorporation since M2 is essential to virus replication. The discrepancy between these two studies may be due to the different scientific approaches taken and the relative size of the F protein domains trying to be incorporated. Influenza M2 is comprised of a 24 amino acid extracellular, 19 amino acid transmembrane and 54 amino acid cytoplasmic domain. Although the transmembrane and cytoplasmic domain between the M2 protein and Sendai virus F protein are similar in size, the extracellular domains are not. It is possible that the virus was unable to incorporate a chimeric protein containing a 499 amino acid extracellular domain which is over 20x the size of the M2e. This would result in data suggesting that the M2e was important for incorporation of the M2 protein into virion particles. However it is also possible that the triple alanine substitution mutations were not sufficient to cause disruption in M2 function. Perhaps more drastic mutations are required for there to be an affect although the M2e was shown to be dispensable for ion channel activity [35].

The M2Stop viruses create a functionally M2-null virus, however they express a truncated version of M2 that only consists of the ectodomain. It is possible that this WT ectodomain is capable of complementing M2e mutants, masking any replication defect associated with the

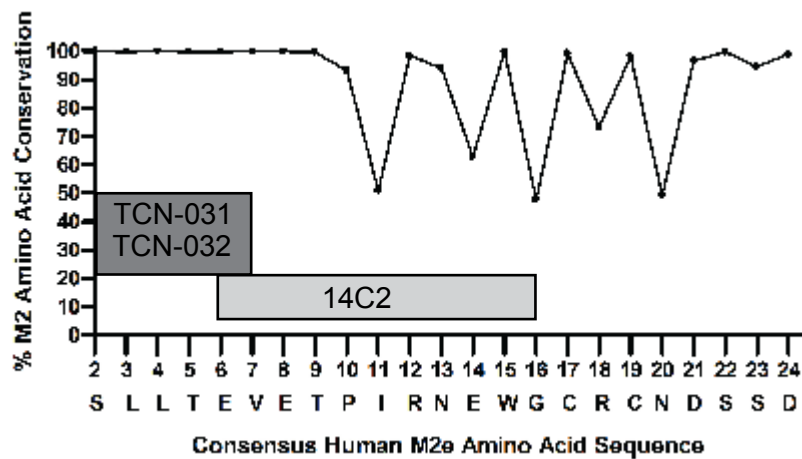
mutations. As WT MDCK cells do not stably express the M2 protein, M2-null viruses would not be expected to replicate. Such complementation is most commonly associated with the lac operon where inactive N-terminal mutant  $\beta$ -galactosidase can return to its active state in the presence of a WT N-terminal fragment of the protein [36]. Analysis of MDCK cells infected with M2Stop viruses have not revealed any small protein fragments that react with 14C2. However if the 24 amino acid ectodomain was still being expressed by M2Stop viruses within cells, it would be hard to detect on a western blot due to its small size. The ability of the WT M2e expressed from M2Stop viruses to complement mutant M2e remains a concern and this data can only be confirmed by mutation of the 24 amino acid ectodomain within the virus itself.

The transcomplementation assay utilized in this study does not demonstrate a requirement of the M2e for M2 function in the viral life cycle, as assessed by virus replication kinetics. Alternative explanations for the high conservation of this region exist but remain to be investigated. HA and NA proteins mutate readily due to immune pressure and thus are poorly conserved. Natural infection with IAV does not induce high titer antibodies to M2 and it is possible that its high conservation is due to the lack of immune pressure. However, studies that have cultured IAV in the presence of M2e antibodies have shown a limited variety of escape mutants, suggesting a functional reasoning for the conserved sequence. The NH<sub>2</sub>-terminal overlap of the M2e to the M1 proteins would suggest a reason for the first eight amino acids to be highly conserved, but the role of first eight amino acids of the M1 N terminus still remains to be determined. The overlap with M1 also does not explain the conservation of the rest of the extracellular domain, which this work showed could be mutated without compromising the core functions of M2.

WSN M2 Constructs	M2 Sequence	Antibody Reactivity		
		14C2	TCN-031	Flag
M2 N31S	mslltevetpirnewgcrndssd	+	+	-
M2 N31S Flag	mslltevetpirnewgcrndssd	+	+	+
2-4 Ala	m <sup>aaa</sup> tevetpirnewgcrndssd	+	-	-
5-7 Ala	msll <sup>aaa</sup> etpirnewgcrndssd	-	-	-
11-13 Ala	mslltevetp <sup>aaa</sup> ewgcrndssd	-	+	-
18, 20-21 Ala	mslltevetpirnewgc <sup>a</sup> <sup>aa</sup> ssd	+	+	-
22-24 Ala	mslltevetpirnewgcrnd <sup>aaa</sup>	+	+	-
5-7 Ala Flag	msll <sup>aaa</sup> etpirnewgcrndssd	-	-	+
8-10 Ala Flag	mslltev <sup>aaa</sup> irnewgcrndssd	-	-	+
14-16 Ala Flag	mslltevetpirn <sup>aaa</sup> crndssd	-	-	+
2-9 Ala Flag	m <sup>aaaaaaaa</sup> pirnewgcrndssd	-	-	+
2-9 Del Flag	m <sup>-----</sup> pirnewgcrndssd	-	-	+

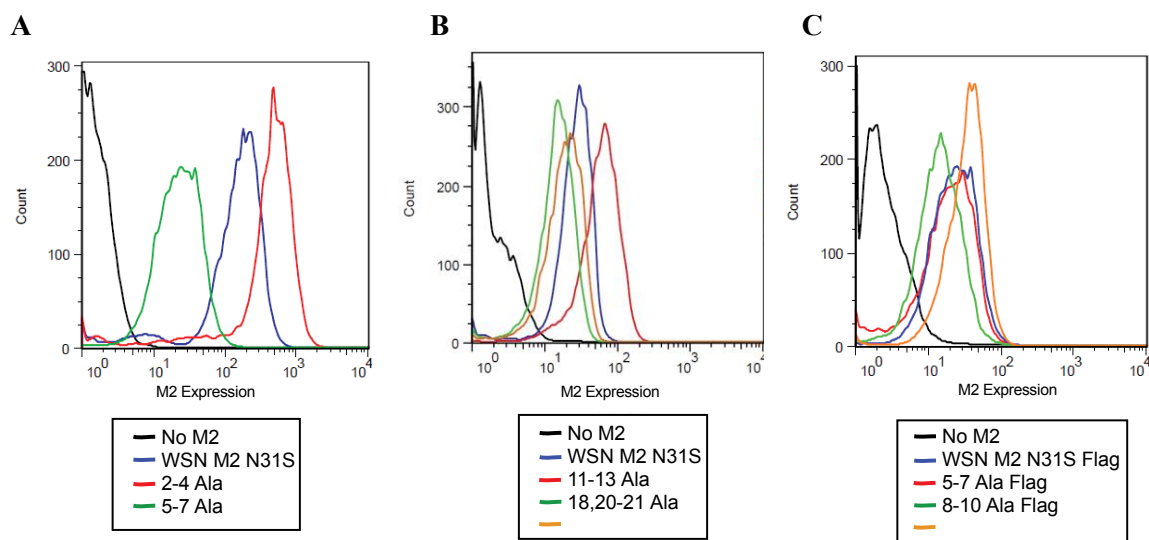
**Table 1: Sequences of WSN M2 extracellular domain and alanine substitution mutants**

Sequence and antibody reactivity of wild-type M2e and mutants. Flag tags are located at the M2 C terminus (sequence not shown) and mutations are highlighted in yellow. 14C2 and TCN-031 are monoclonal M2 antibodies and flag denotes a monoclonal anti-flag antibody.



**Figure 1: Conservation of the M2e amino acid sequence.**

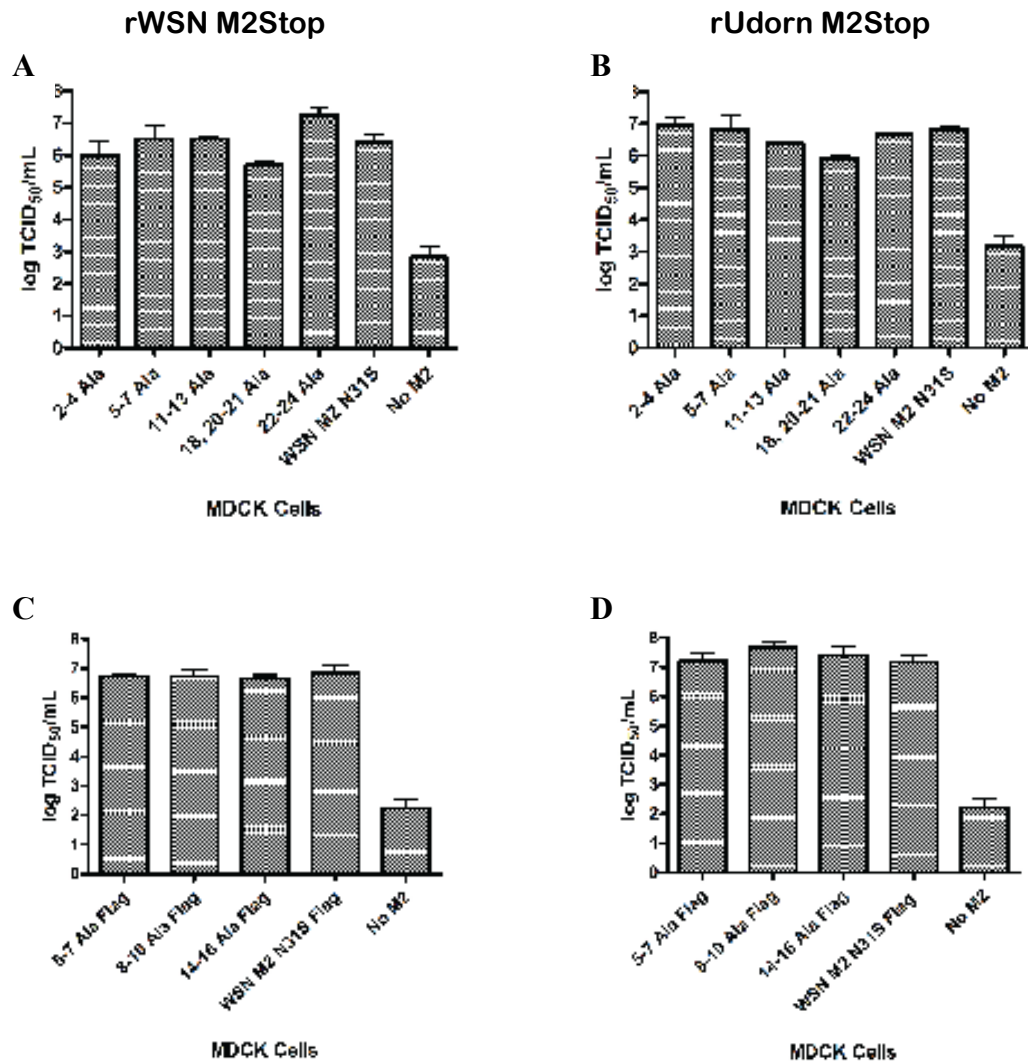
Conservation of the M2e amino acid sequence, from residue 2 to 24, was compiled from all influenza A virus strains. The percent M2e conservation was plotted against the consensus human M2e amino acid sequence on the x axis. Epitope regions were mapped for the M2 monoclonal antibodies TCN-031, TCN-032 and 14C2.



**Figure 2: Analysis of clonal expression levels of the M2 protein in stable MDCK cell lines.**

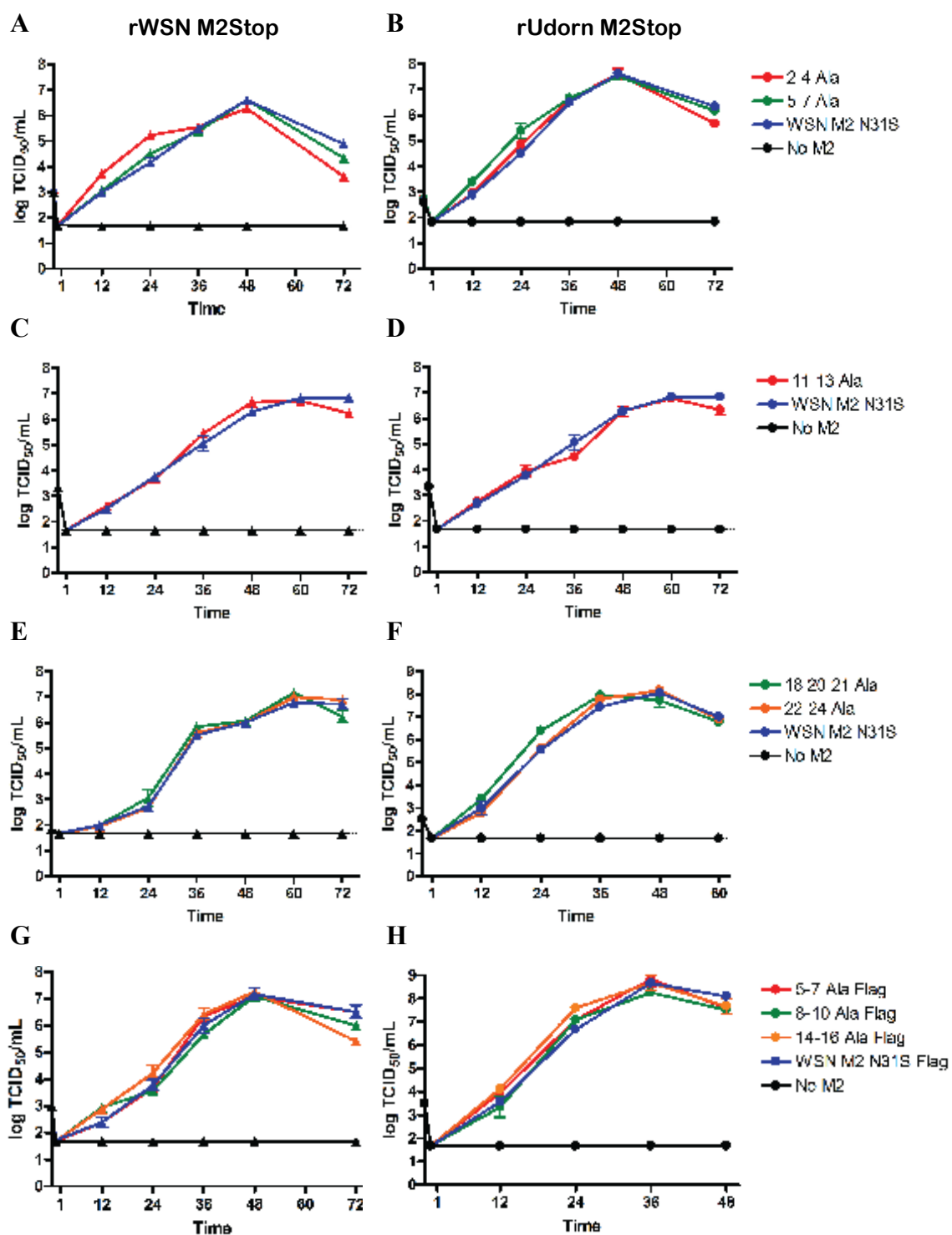
The clonal expression levels and number of cells expressing M2 at the cell surface were analyzed using monoclonal M2 antibodies (A) 14C2, and (B) TCN-031. (C) Expression levels of cell lines with an M2 C-terminal flag tag were permeabilized and detected using an anti-flag antibody. Each plot is cell number vs increasing M2 expression.





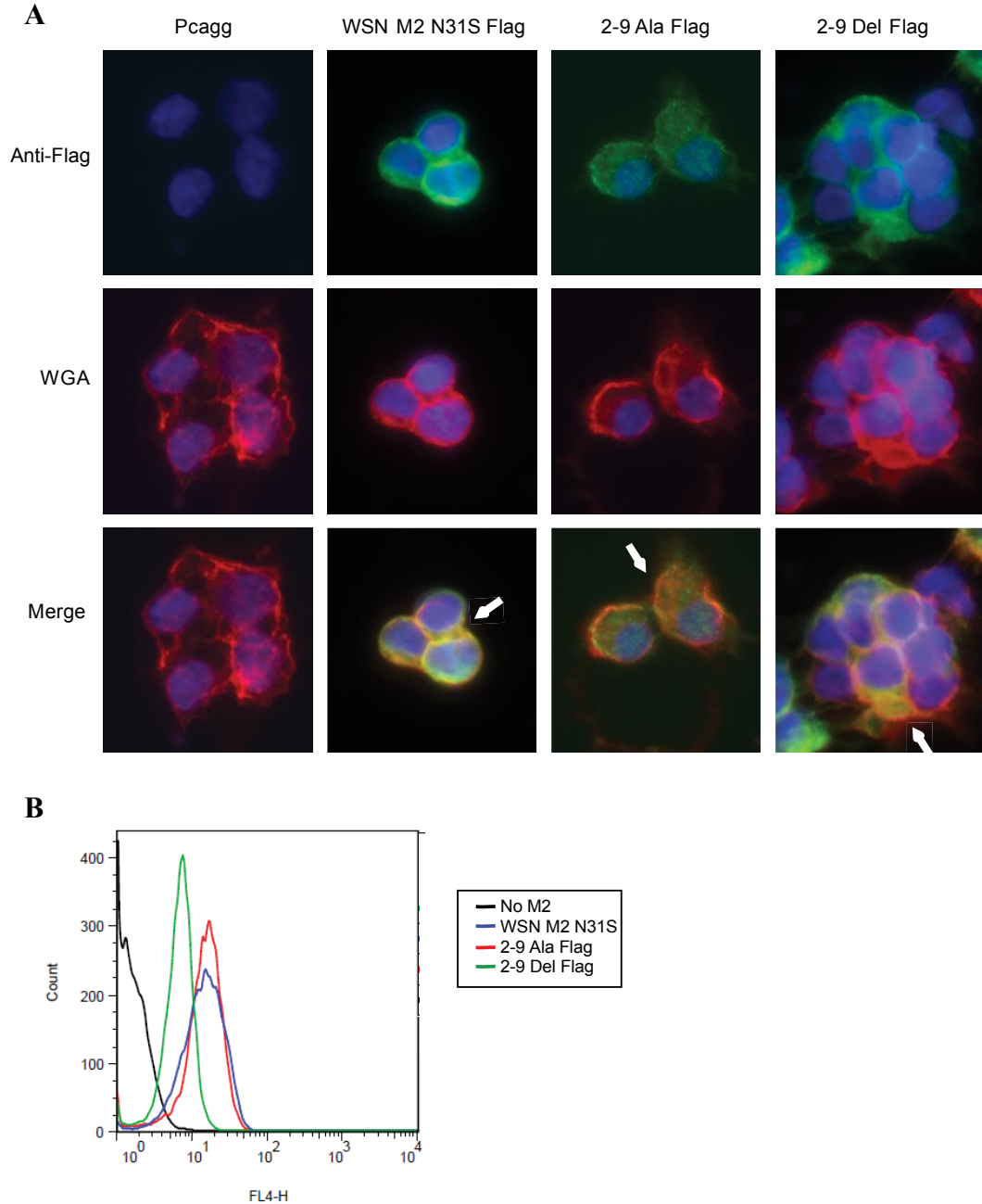
**Figure 3: Mutating the M2e does not affect the ability to complement a M2-null virus via a TCID<sub>50</sub> assay.**

Titers of rWSN M2Stop (A and C) and rUdorn M2Stop (B and D) were determined for each indicated cell line by TCID<sub>50</sub> assay. The mean and standard error of the mean are graphed from three independent experiments. Total infectious virus production was analyzed by one-way ANOVA using Prism 4.0. Statistical differences between M2 expressing cell lines and no M2 are indicated. \* p<0.05



**Figure 4: Mutation of the M2e does not affect complementation growth kinetics of M2 stop viruses as compared to WSN M2 N31S.**

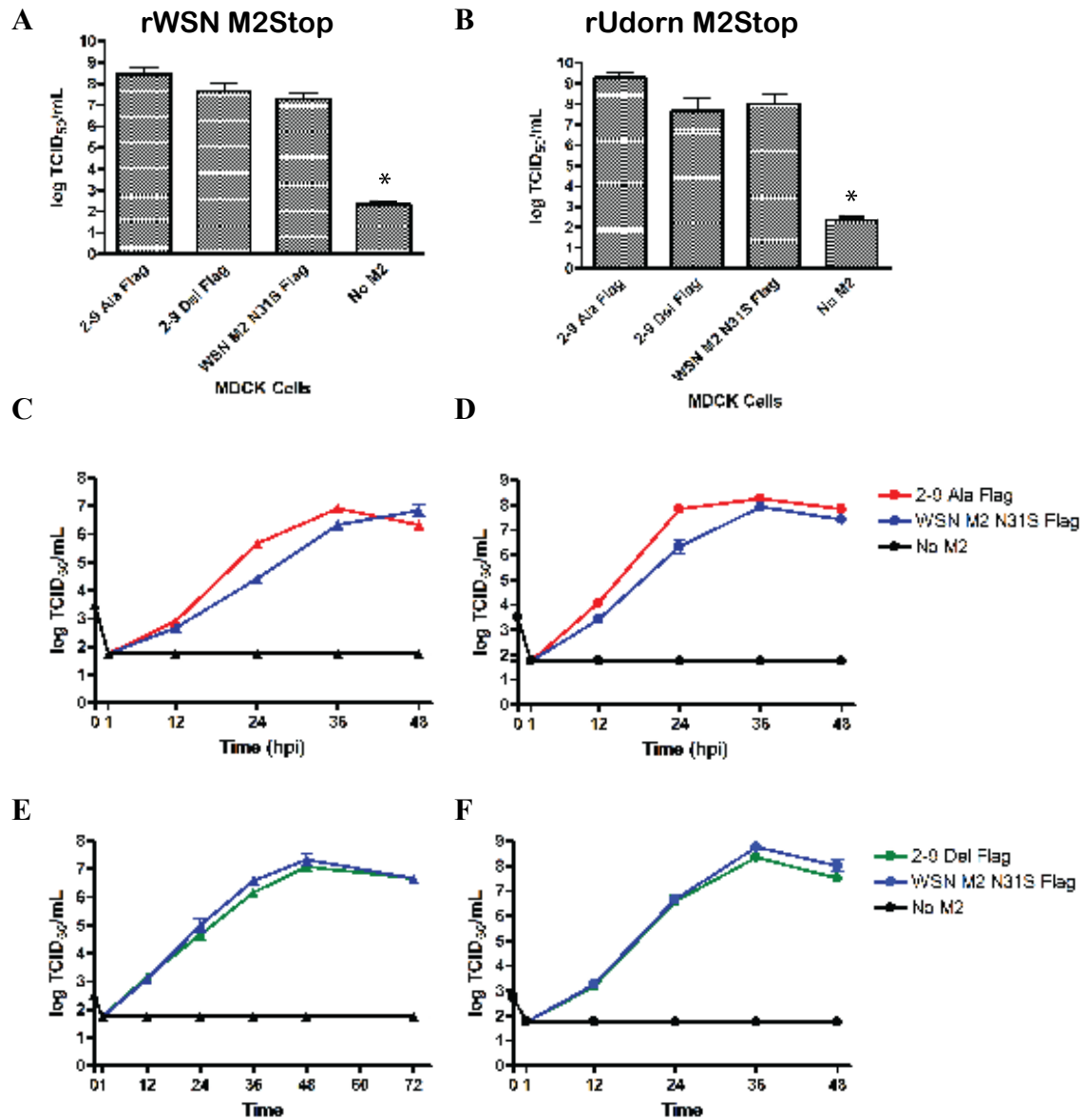
MDCK cells expressing the indicated M2 protein were infected at a low MOI of 0.001 with either rWSN M2Stop (A, C, E and G) or rUdorn M2Stop (B, D, F and H) to determine the effect of the M2e mutations on growth kinetics of homologous (rWSN) and heterologous (rUdorn) viruses. The amount of infectious virus at each time point was determined by a TCID<sub>50</sub> assay on WSN N31S M2 cells. The mean and standard error of the mean of triplicate samples are graphed from a representative experiment and the limit of detection is marked by a horizontal dotted line.



**Figure 5: Expression and cell surface localization of 2-9 Ala and 2-9 Del Flag.**

(A) Transfection of 293T cells with pCAGG empty vector (left column), pCAGG WSN M2 N31S (second column), pCAGG 2-9 Ala Flag (third column) and pCAGG 2-9 Del Flag (right column) plasmids. Cells were stained with anti-flag (first row) to detect M2 tagged constructs, wheat germ agglutinin (WGA; middle row) as a plasma membrane marker and merged (bottom

row) images. Colocalization was determined from 10 non-overlapping images taken with an epifluorescence microscope. One representative image is shown with colocalization indicated by white arrows. (B) Clonal expression levels of M2 in stable MDCK cell lines expressing 2-9 Ala Flag and 2-9 Del Flag were determined by flow cytometry. Cells were permeabilized and stained for flag using a monoclonal anti-flag antibody. Cell number was plotted against increasing M2 expression.



**Figure 6: Mutation of the highly conserved first nine amino acids of M2e does not affect complementation or growth kinetics of M2 stop viruses as compared to WSN M2 N31S.**

The TCID<sub>50</sub> for rWSN M2Stop (A) and rUdorn M2Stop (B) were determined for 2-9 Ala Flag and 2-9 Del Flag. The mean and standard error of the mean are graphed from three independent experiments. Total infectious virus production was analyzed by a one-way ANOVA using Prism

4.0. Statistical differences between M2 expressing cell lines and no M2 are indicated. \*  $p < 0.05$

MDCK cells expressing the indicated M2 protein were infected at a low MOI of 0.001 with either rWSN M2Stop (C and E) or rUdorn M2Stop (D and F) to determine the effect of the M2e mutations on growth kinetics of homologous (rWSN) and heterologous (rUdorn) viruses. The amount of infectious virus at each time point was determined by a TCID<sub>50</sub> assay on WSN N31S M2 cells. The mean and standard error of the mean of triplicate samples are graphed from a representative experiment and the limit of detection is marked by a horizontal dotted line.

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## **Chapter Three:**

### **Prospects for oral replicating adenovirus-vectored vaccines**

Adapted from: Deal C, Pekosz A, Ketner G. Prospects for Oral Replicating Adenovirus-Vectored Vaccines. *Vaccine*. 2013; 31 (32): 3236-43

## Introduction

Oral delivery of immunogens to the gut is regarded as the “Holy Grail” for vaccinologists [1]. The intestine is the largest lymphoid organ and gut-associated immune cells represent up to 90% of immunocompetent cells [2]. Oral immunization offers immunological and logistical advantages including stimulation of mucosal immune responses preferentially at the site of entry for many infectious agents and ability to elicit strong systemic immunity. This immunization route is cost effective and offers improved patient compliance due to the ease of vaccine administration, freedom from needles and from the requirement for trained medical personnel. All three oral vaccines licensed for use in the US [3] contain live virus. Live-virus vaccines add to the inherent advantages of oral immunization the ability to immunize with small (and hence less expensive) doses, and induction of a breadth of immune responses similar to those induced by natural infection. These characteristics would facilitate routine immunization and response to epidemics or pandemics [4] and make live oral vaccine immunization attractive in resource-poor regions, where economy and logistical tractability are critically important.

Licensed oral adenovirus (Ad) serotype 4 and 7 vaccines provide a model for use of live recombinant adenoviruses (rAds) for oral immunization. Since the 1970's, live oral Ad vaccines have been used by the United States military to prevent acute respiratory disease caused by Ad4 and 7 [5]. These vaccines contain lyophilized live, wild type (WT) virus incorporated into enteric tablets that protect the virus against the low pH of the stomach. After oral administration of the tablets, live virus is released into the intestine where asymptomatic replication occurs. In a single dose, the vaccines generate an immune response that was over 95% effective in preventing Ad4- and Ad7-induced respiratory illness in a clinical trial involving more than 40,000 soldiers [6-9]. The historical success of Ad military vaccines suggests great potential for recombinant vaccines using the oral replicating Ad platform.

rAds have been used to deliver vaccine antigens in over 90 pre-clinical and clinical trials [10, 11]. The rationales for use of rAd vaccines include genome stability and ease of manipulation, natural tropism for mucosal inductive sites including the gut and upper respiratory tract and ability to elicit vigorous humoral and cellular immune responses. rAds infect a broad spectrum of cells, including dendritic cells, allowing for efficient antigen presentation and can therefore also prime a robust cell-mediated response [12, 13]. Most rAd vaccine candidates are replication defective and not intended for oral administration. Here, we review work on replicating rAd vaccines that may provide a route to effective oral immunization.

### **Replicating rAd transgene vectors as vaccines**

Most current rAd vaccine candidates are transgene expression vectors, commonly engineered to express a foreign gene inserted into early region 1 (E1) or, occasionally, early region 4 (E4) of the genome [14]. E1 and E4 are essential for viral replication, and most such rAds are replication-defective. Extensive experience with defective recombinants in humans and animal models has shown promise in several cases [15].

Replication-competent transgene vectors can be constructed by careful choice of the site of transgene insertion but relatively few have been extensively investigated. Study of replicating rAd vaccines is complicated by the requirement for a host that supports viral replication if vaccines are to be evaluated under conditions that mimic its intended use in humans. Mice do not support human adenovirus replication. However, golden hamsters, cotton rats, dogs, pigs, monkeys (see below), and chimpanzees all support replication of some human Ads, providing systems that might be exploited to test replicating vaccines [16-21]. Cotton rats and guinea pigs have found use in characterization of replicating oncolytic adenoviruses [16, 22], and dogs have been used in evaluation of live rAd vaccines [18]. In practice, however, well-developed immunological reagents, perceived similarity of primate and human immune responses, and

availability of suitable challenges to assess efficacy have restricted most studies of replication-competent rAds in permissive hosts to primates (chimpanzees or monkeys), or to human volunteers.

In early studies, replication-competent rAd7 and rAd4 expressing the hepatitis B virus surface antigen (HBsAg) were used to immunize (rAd7 HBsAg) and then boost (rAd4 HBsAg) two Ad4, Ad7-seronegative chimpanzees (rAd7/rAd4 HBsAg) by the oral route [20]. After primary vaccinations, both chimpanzees shed vaccine virus for 6-7 weeks and developed Ad7 antibodies, suggesting successful Ad7 replication in the chimpanzee gut. One developed transient seropositivity for HBsAg after the first inoculation; both developed modest titers after the second. A third chimpanzee immunized with WT Ad7 and then rAd4 HBsAg (WTAd7/rAd4 HBsAg) developed no HBsAg antibodies. Both rAd7/rAd4 HBsAg chimpanzees were protected from acute clinical disease but were not protected from infection as evident by development of antibodies against the HBV core protein in response to HBV challenge. The animal that did not seroconvert (WTAd7/rAd4 HBsAg), along with an unimmunized control, became clinically infected with HBV [20]. Three human volunteers in a small phase I vaccine trial immunized with the rAd7 HBsAg vaccine exhibited no adverse effects and shed virus between days 4 and 13 post vaccination with no evidence of person-to-person spread. Although all subjects had a significant increase in Ad7 antibodies, none made antibodies to HBsAg [23]. Protection from disease, if not infection, in chimpanzees, despite lack of seroconversion in humans, suggests potential value in using oral enteric vaccination with rAd to induce humoral immune responses to foreign pathogens.

Most animal studies of replicating rAds have been conducted in macaques. WT Ad2 and Ad5 do not replicate in monkeys, and these experiments therefore require use of an Ad5 host range mutation (*hr404*), located in the 72k DNA binding protein, that permits replication in monkey cells and macaques [21, 24]. A transgene-type rAd5 *hr404* (rAd5hr) virus expressing the



*env* and *rev* genes from SIV (Ad5hr-SIV*env/rev*) was able to replicate *in vivo* in rhesus macaques [25]. Priming orally and intranasally, followed by intratracheal immunization 12 weeks later with Ad5hr-SIV*env/rev*, generated proliferating T cells to *env* and strong serum neutralizing anti-*env* antibodies. Mucosal secretions also contained *env*-specific IgG and IgA antibodies. Although this vaccine did not induce sterilizing immunity, it conferred acute-phase protection following intravaginal challenge with SIV [25]. Partial protection of reboosted and rechallenged transiently viremic macaques was associated with both cellular and humoral immune responses [26]. To broaden rAd-induced immunity to SIV, additional rhesus macaques were immunized simultaneously with replicating constructs expressing SIV *env*, *rev* and *gag* through oral and intranasal administration [27]. Specific T-cell responses were generated against all SIV gene products and there was a persistent response to Gag evident for more than 10 weeks post-immunization. Interestingly, immunization primed CD8+ T cells for a persistent and potent response to both dominant and subdominant epitopes [27, 28]. Intrarectal challenge with SIV demonstrated that the vaccine did not induce sterile immunity but acute viral replication was suppressed. Cellular immunity to SIV Gag and Env, along with nasal and vaginal *env*-specific IgG antibodies, correlated with a significant reduction of acute phase viremia [29]. Immunized groups exhibited significant protection, with 39% of macaques having either no viremia, cleared viremia or controlled viremia at the threshold of detection 40 weeks post-challenge.

In these studies, only 35% of macaques exhibiting rAd shedding [27], suggesting that the protocol used, bicarbonate neutralization of the stomach prior to virus delivery, might not preserve rAd infectivity. Use of enteric-coated capsules for virus administration resulted in shedding virus in stool samples of 100% of immunized macaques [30] emphasizing the importance of an optimal oral delivery method.

Recently, phase I clinical trial data has been presented for a transgene-type replication-competent rAd4 vaccine (rAd4-H5-Vtn) expressing influenza H5 hemagglutinin (HA) [31]. This

virus, which induced protective immune responses in a nonpermissive mouse model [32], contains an insertion of the H5 HA gene in place of part of E3. 166 healthy volunteers received vaccine dosages ranging from  $10^7$  to  $10^{11}$  recombinant virus particles (VP) [31]. Each cohort received three rAd vaccinations orally and an intramuscular boost with inactivated H5N1 vaccine. Administration of the rAd was associated with significantly increased mild headache, abdominal pain, nasal congestion and diarrhea, and there was no confirmed transmission of the rAd4-H5-Vtn virus to household contacts. Pre-existing antibody to Ad4 was associated with a lower immune response to the vaccine, but this effect was overcome in the high-dose cohorts of  $10^{10}$  and  $10^{11}$  VP. In mice, this recombinant elicits good humoral Ad4 and HA responses but a low cell-mediated response [32]. In humans, the vaccine induced a significant level of Ad4 seroconversion and HA-specific cellular immune responses in 70% of volunteers receiving  $10^{11}$  VP [31]. However, HA-specific antibody responses assessed by hemagglutination-inhibition (HAI) were minimal at all doses tested, with seroconversion in 4% to 19% of vaccinated volunteers. Plasma IgA ELISA titers mirrored HAI, although IgG ELISA responses indicated 50% seroconversion in the  $10^{11}$  VP cohort. The H5 HA antigen is an intrinsically poor immunogen [33], however following boost of the inactivated H5N1 vaccine, 80% to 100% of volunteers seroconverted and 80% to 89% demonstrated antibody titers high enough to be considered protective in the  $10^{10}$  and  $10^{11}$  VP cohorts, respectively [31]. This indicates that although the Ad4-H5-Vtn vaccine can induce a cellular response, it is only capable of priming an HA-specific antibody response. The cellular immune response and replication of the vaccine as assessed by Ad4 seroconversion or PCR positive rectal swabs, primarily occurred after the first dose, suggesting that only one oral dose may be necessary to induce a cellular response and prime an antibody response.

The doses required in this study to induce vector immune responses are 100-fold (or more) greater than that in the Ad4 vaccine ( $10^5 - 10^7$  TCID<sub>50</sub> [5]). rAd4-H5-Vtn lacks E3, which

functions in evading the host immune response [34] and may play an important role in the immunogenicity and safety of replicating rAd vaccines. That possibility has not been experimentally addressed.

Numerous clinical trials of replicating oncolytic rAds have been conducted. In general, these studies do not include analyses of immune responses. Where Ad responses have been measured they are efficiently induced [35, 36], but there are no reports of responses to transgene products.

### **Replicating capsid display rAds as vaccines**

Despite the efficacy of the oral Ad4 and Ad7 vaccines and efficient induction of antibodies against the vector, oral rAd vectors induce only modest antibody responses to transgene products in both replicating and non-replicating forms [20, 37] (Berg and Ketner, unpublished). However, a second rAd antigen expression method may offer a more potent approach to induction of humoral immunity. In capsid-display recombinants, segments of foreign antigens are incorporated into one of the capsid proteins such that they are displayed on the surface of the virus particle. Capsid-incorporated antigens are available for binding by surface antibody on B cells and can be processed by the exogenous (MHC class II) pathway. Thus, capsid display recombinants can be immunogenic without intracellular antigen expression, including in systems that do not support virus replication. Replication in a permissive host would further allow persistent antigen presentation via both the exogenous and the endogenous (MHC class I) pathways, with the potential of inducing both humoral and cellular responses. Capsid-display vectors are extremely immunogenic in mice [38-40] and therefore may offer greater efficacy in inducing humoral responses in permissive systems than do transgene rAds.

Several capsid proteins can display foreign epitopes, including hexon, fiber, penton base and pIX (Table 1, Figure 1A, and below). Currently, immunogenicity data is available only in mice, and conclusions therefore have been drawn only in the absence of viral replication.

**Hexon (polypeptide II)** The ~960 amino acid Ad hexon protein is the most abundant of the capsid proteins, present in 720 copies per particle [41]. Analysis of hexon amino acid sequences from different serotypes revealed 9 hypervariable regions (HVRs) that diverge in sequence and length among serotypes [42]. Crystal structures of Ad2 and Ad5 hexon show that HVRs reside in two loops that form the surface-exposed portion of hexon. HVR 1-6 are located within the DEI loop and HVR 7-9 lie within the FGI loop (Figure 1 B and D) [42, 43]. These HVRs contain serotype-specific epitopes that are primary targets of neutralizing antibodies (nAb) [44].

X-ray crystallography suggests that HVRs 2, 3, 5, 6 and 7 are unordered and protrude from the capsid surface. Ad5 virus containing insertions of His<sub>6</sub> peptides with flanking spacers into those HVRs are viable, with normal virion thermostability and infectivity [45]. His<sub>6</sub> in HVR2 or 5 is capable of binding tightly to the His<sub>6</sub> antibody, suggesting that the tag is exposed on the virion when incorporated into these regions [45]. Assessed with epitopes of increasing size, HVR5 was found to accommodate a maximum of 65 amino acids, while the maximum length accommodated in HVR2 was 33 amino acids [46]. While HVR1 of Ad5 has been shown to accommodate up to 24 amino acid insertions [47], the insertion of only 17 amino acids resulted in viable virus in Ad3 HVR1 [48]. Modifications in HVR1 or 5 reduced susceptibility to neutralization by preexisting immunity (PEI) to the Ad vector [38, 49]. Substituting all the HVR loops in Ad5 with those derived from Ad43, a serotype with a low seroprevalence in humans, produced a vector capable of escaping neutralization with anti-Ad5 sera from mouse, rabbit and humans [50] and which was still highly immunogenic in the presence of PEI to the WT virus.

The first capsid display recombinants incorporated 8 amino acids of the poliovirus type 3 VP1 capsid protein into regions now recognized as HVR1/2. Antiserum raised against the rAd recognized the poliovirus epitope on the Ad virion and the poliovirus capsid itself [51]. Worgall *et al.* incorporated an immunodominant peptide from the outer membrane protein F (OprF) of *Pseudomonas aeruginosa* into HVR5 [40]. Immunization with this rAd induced IgG1 and IgG2a antibody subtypes, elicited epitope-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses, and was capable of protecting 60-80% of mice from a lethal pulmonary challenge with three different *P. aeruginosa* strains. Efficacy was increased with subsequent boosts [40, 52]. In contrast, a B-cell epitope from *Bacillus anthracis* protective antigen (PA), a subunit of the lethal toxin, incorporated into HVR5, induced non-neutralizing antibodies and failed to protect against a challenge with lethal toxin [53]. The discordant results from these studies may reflect differential antibody titers or differing properties of the selected epitopes.

Subsequently, Shiratsuchi *et al.*, inserted a B cell epitope from the circumsporozoite protein (CSP) of the murine malaria parasite *Plasmodium yoelii* into hexon HVR1 or 5 in a recombinant that also expressed CSP as a transgene [38]. The HVR1 recombinant induced high titer antibodies even in mice pre-immunized with WT Ad, suggesting that alteration of HVR1 allowed for evasion of neutralizing Ad antibodies. An rAd incorporating a B-cell epitope from *P. falciparum* CSP in HVR1 induced high-titer antibodies in mice that recognized parasites expressing the *P. falciparum* CSP and neutralized sporozoites bearing the *P. falciparum* CSP gene *in vitro* [47].

The location of epitopes inserted in hexon is an important determinant of immunological properties [46]. rAds that displayed an epitope from the VP1 capsid protein of Enterovirus 71, in HVRs 1, 2, or 7 were viable and protected neonatal mice from lethal challenge through passive immunization and maternally-acquired antibodies [54]. However, antibody isotype depended on the location of the epitope insertion: insertions into HVR1 induced mostly IgG2a antibodies

(Th1) while an HVR7 insertion predominantly produced IgG1 antibodies (Th2), demonstrating that insertion sites on hexon are not immunologically equivalent [54]. Similarly, of insertion of the conserved extracellular domain of matrix protein 2 (M2e) of influenza A virus into variable region 1 (VR1) or VR4 of hexon of the chimpanzee-origin adenovirus SAd-V25 (AdC68), only the VR1 recombinant provided partial protection from a lethal influenza challenge [55]. Capsid display recombinants induced more robust responses than a transgene type recombinant expressing an M2e fusion protein, supporting the hypothesis that antibody responses are best induced by antigen displayed in a repetitive and structured fashion to allow for cross-linkage of the B cell receptors [56].

Recent studies of rAds with modifications in two hexon HVRs have demonstrated the potential for single recombinants to elicit simultaneous antibody responses against two distinct epitopes [57]. ‘Multivalent’ capsid display recombinants offer potential for broadening immune responses or inducing responses to genetically variable pathogens. However, recombinants with different combinations of modified HVRs induced strikingly different responses, indicating that the design of effective multivalent hexon-modified rAds may not be straightforward [57]. While Gu *et al* [57] utilized multiple HVRs to insert epitopes, Zhong *et al* attempted to incorporate multiple epitopes into a single HVR [48] and found that antiserum was raised against a combination of the epitope and not against the individual epitopes themselves. Thus, replacement of several HVRs is a more promising alternative to a polyvalent insertion to generate multivalent vaccine vectors.

**Penton base (polypeptide III)** The penton base and fiber form the penton complex present at the 12 vertices of the capsid (Figure 1A). Each penton base monomer (~570 residues) contains an Arg-Gly-Asp (RGD) integrin-binding motif located within a flexible loop at the capsid surface [58]. An influenza A virus HA epitope inserted into the RGD loop of penton base was accessible to anti-HA antibodies, confirming surface location [59]. However, anti-HA antibodies were not

detected in mice immunized with a penton base recombinant containing HA inserted into the RGD loop [59]. The insertion decreased infectivity for DC's, potentially by interfering with integrin binding, which is involved in virion internalization.

**Fiber (polypeptide IV)** Fibers are homotrimers of the fiber protein (polypeptide IV) that protrude from the 12 vertices of the Ad virion and are responsible for attachment to the host cell (Figure 1A). The fiber protein has 3 domains: an N-terminal domain that attaches to the penton base, a central shaft with repeating motifs, and a C-terminal globular knob responsible for virus attachment to the host cell (Figure 1E). Ad5 fiber contains 582 amino acids and is 35-40 nM in length, but fiber length varies among serotypes due to differing numbers of repeats in the fiber shaft.

The crystal structure of the fiber knob reveals that the HI loop (Figure 1C and 1E) does not contribute to intramolecular interactions within the knob, consists mostly of hydrophilic amino acid residues, is exposed on the surface of the knob and is not involved in the formation of cell-binding sites [60]. A FLAG epitope inserted into the HI loop was also accessible to anti-FLAG antibodies, confirming that the HI loop is exposed [61]. Therefore, the HI loop is seen as particularly suitable for manipulation and most modifications initially were made at this location [59, 62]. More recently, a series of rAds with insertions of the *P. aeruginosa* OprF Epi8 epitope in fiber loops CD, DE, FG, HI and at the C terminus [63] have been examined for effects of insertions on viral growth *in vitro* and for immunogenicity. Incorporation of Epi8 into the FG and HI loops had little effect on viral growth whereas insertion into the CD and DE loops or at the C terminus strongly reduced infectivity. FG and HI loop insertions also elicited the strongest humoral and cell-mediated immune responses and were partially protective against challenge [63].

Fiber is a target for neutralizing antibodies and substitutions can contribute to evasion of PEI. For example, modification of the HI loop circumvented nAb present in ascites fluid from ovarian cancer patients [64]. Consistent with this, FG and HI loop recombinants were more effective at inducing antibody and protection in the presence of PEI than was a transgene-type recombinant expressing all of OprF [63]. The ability to manipulate fiber at multiple sites to allow for the efficacy in the presence of PEI makes fiber insertions a promising modification for capsid-display vaccines.

Fiber modifications intended to redirect or ablate virus binding to specific cellular receptors have also been explored [40, 65-67]. However, immunogenicity generally is not addressed in those studies.

**pIX (polypeptide IX)** pIX (approximately 140 amino acids) is present in about 240 copies per virion. Trimers of pIX contribute to stability of the virus particle [68, 69]. The C-terminus of pIX is exposed on the surface of the virion and has been used as a substrate on which to attach large polypeptides including fluorescent proteins, fully functional enzymes and foreign antigens in viable rAds [70-74]. pIX fusions containing the envelope protein gp70 of the Friend murine leukemia virus (FV) [74] and the *Yersinia pestis* V and F1 capsular antigens [39] induced high-titer antibodies. The ability of pIX to accommodate very large proteins makes it an attractive site for display of conformational epitopes.

**Comparative immunogenicity.** The immunogenicity of influenza A virus HA epitopes inserted into various Ad capsid proteins has been compared [59]. Insertion sites included hexon HVR5, the RGD loop of penton base, the HI loop of the fiber knob and the C terminus of pIX. All HA insertions were located on the virion surface, however, an anti-HA antibody demonstrated strongest binding to HA incorporated into hexon. Infection of A549 cells and DCs showed that HA incorporation into hexon interferes minimally with virus entry *in vitro*, whereas incorporation



into fiber knob, pIX and penton base partially reduced the intracellular Ad genome copy numbers following infection. The humoral immune response was strongest against the hexon insertion when immunizing with the same number of particles but fiber was the most immunogenic when controlling for the number of HA copies per virion [59]. A comparison of an ovalbumin (OVA) epitope inserted into the fiber HI loop or hexon HVR5 indicated that fiber insertions were better detected in native virions and triggered a more dramatic increase in anti-OVA antibody responses upon re-administration [62].

**Pre-existing Immunity and replicating rAds.** Antibodies to many Ad serotypes are prevalent in the human population. PEI to the vaccine serotype can interfere with a robust immune response against the foreign antigen even in non-replicating rAds [20, 75], although mucosally administered replication-defective rAd vaccines have elicited transgene-specific antibodies despite the presence of PEI, and homologous serotype boosts can be effective [29, 37, 76]. Importantly, if capable of suppressing the growth of viable rAds, PEI might mitigate the inherent advantage of vaccine vector replication after administration of a low dose [77], and live rAds thus may be more sensitive to PEI than their defective counterparts. PEI can be addressed by use of uncommon human adenovirus serotypes or viruses from other species [37, 78, 79] as vectors. Additionally, as noted above, modifications to both hexon and fiber have been shown to reduce susceptibility to PEI [38, 49, 50] and properly-designed capsid display rAds therefore may be inherently resistant to PEI. Limited experience with replicating rAds has provided no data on the effects of PEI and this topic must be addressed.

**Safety of replicating rAds.** Concerns have been expressed over the safety of replicating vaccines due to the possibility of inducing disease in the immunocompromised and to the possibility of unintentional spread to contacts. Systemic adenovirus infections can be fatal in people who are profoundly immunocompromised, for example, in the course of bone marrow transplantation [80, 81]. Further, Ad is commonly present in AIDS-related deaths, although it is not generally

believed that it was the cause [82, 83]. Clearly, live rAds cannot be administered to the severely immunodeficient. However, unwitting administration of the military vaccine to a small number of recruits with early HIV infection produced no observed ill effects, nor did the concurrent HIV infection prolong shedding, suggesting a small margin of safety in that population [84].

Transmission of the oral military Ad vaccine did occur, but required intimate contact, as it was not observed among recruits in the barracks [85, 86], and no confirmed transmission of the rAd4-H5-Vtn virus to contacts in its recent clinical trial [31]. Thus, the hazard associated with live vaccines may not be insurmountable, although this aspect of use of live vaccines, rAd or others, must be carefully investigated.

## **Conclusion**

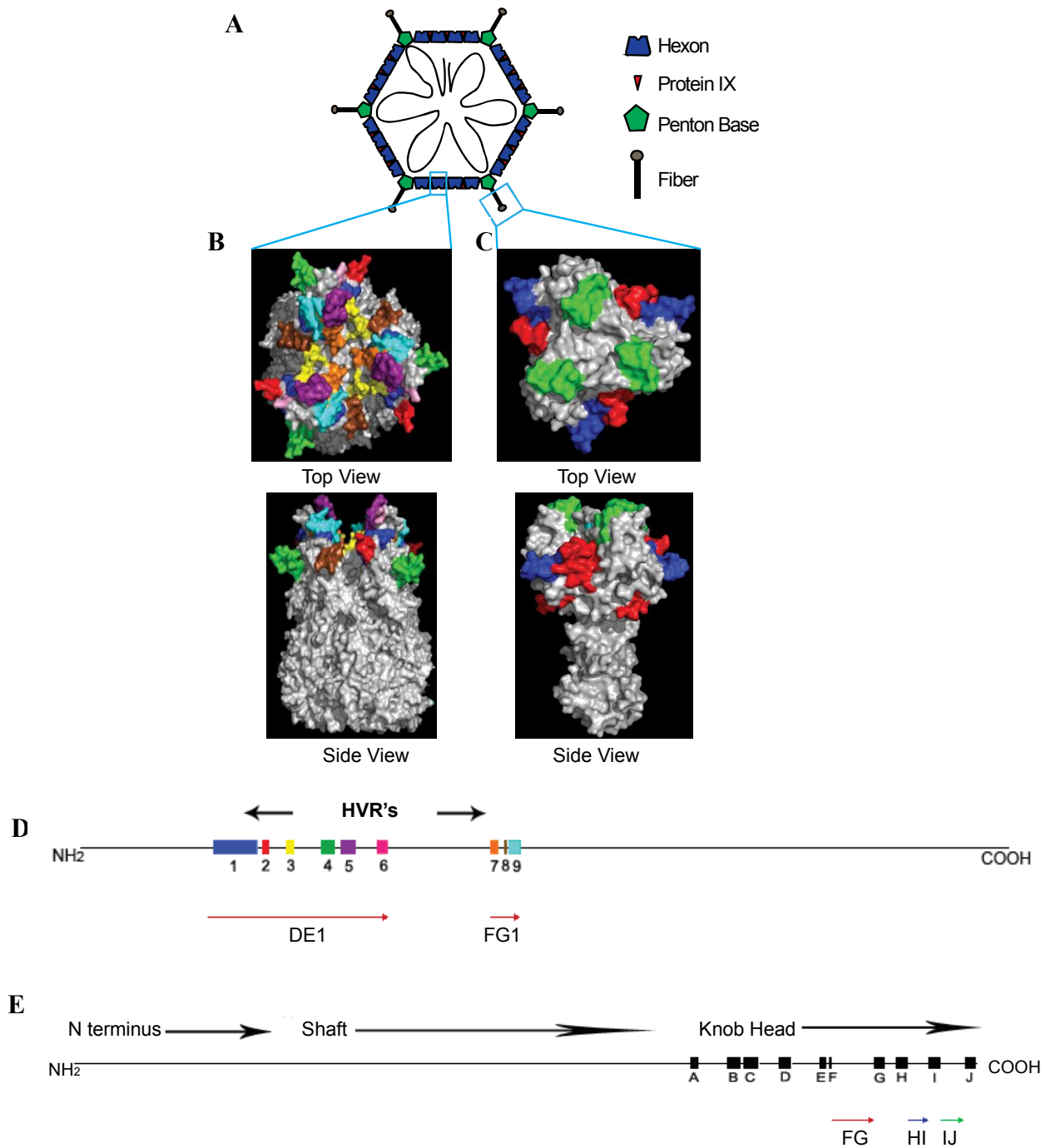
Replication-competent transgene or capsid display rAds delivered orally to the gut mucosa offer an unconventional immunization approach. Transgene rAds have been shown to induce a robust cellular-mediated immune response, and capsid-display rAds promise to induce strong humoral responses. Critically, transgene and capsid display designs possess complementary immunological characteristics and can be combined in single rAds [38], and such hybrid rAds offer a potential route to greater potency than either approach alone. Multiple-antigen hybrid rAds, in particular, may be capable of increasing the breadth of immune responses to pathogens with a high mutation rate, such as influenza A, or a complex biology, such as malaria. Continued innovation in vaccine research is critical in order to control diseases such as HIV, influenza and malaria that have proven resistant to conventional immunization strategies, and replicating rAds have earned their place among the novel strategies worthy of exploration.

<b>Capsid Protein</b>	<b>Insertion Sites</b>	<b>Maximum Foreign Epitope Insertion Size<sup>b</sup></b>	<b>References</b>
Hexon	HVR1	24	[38, 47-51, 54, 57]
	HVR2	45	[45, 46, 50, 51, 54, 57, 87, 88]
	HVR3	6	[45, 50]
	HVR4	15	[50, 54]
	HVR5	66	[40, 45, 46, 49, 50, 52-54, 57, 59, 62, 89]
	HVR6	6	[45, 50]
	HVR7	15	[45, 50, 54]
	HVR8	--	[50]
	HVR9	--	[50]
	VR1 <sup>a</sup>	24	[55]
	VR4 <sup>a</sup>	24	[55]
Penton	RGD loop	9	[59]
Fiber	C terminus	14	[63, 90-92]
	CD loop	14	[63]
	DE loop	14	[63]
	FG loop	16	[63, 93]
	HI loop	22	[40, 59, 61-67, 93]
	IJ loop	16	[93]
pIX	C terminus	393	[39, 59, 70-74, 94]

**Table 1: Capsid protein insertion sites**

<sup>a</sup> non-human adenovirus serotype with only 5 variable regions

<sup>b</sup> size in amino acids



**Figure 1: Adenovirus capsid structure**

(A) Cartoon diagram of an adenovirus particle depicting capsid proteins and DNA. (B) Surface model of the trimeric Ad5 hexon protein showing the HVR regions. The amino acid location of the region is located in parenthesis. Blue: HVR1 (137-181), red: HVR2 (187-193), yellow:

HVR3 (211-218), green: HVR4 (247-260), purple: HVR5 (267-282), brown HVR6 (304-315), orange: HVR7 (418-428), pink: HVR8 (435-436), cyan: HVR9 (440-451). Produced using program PYMOL and PDB 3IYN [95]. (C) Surface model of the trimeric Ad2 penton knob and shaft. Red: FG loop (488-515), blue: HI loop (537-549), green: IJ loop (558-572). Produced using program PYMOL and PDB 1QIU [96]. (D) Map of the Ad5 hexon protein with labeled HVRs. (E) Map of the fiber protein with labeled  $\beta$ -strands in black boxes and FG, HI and IJ loops indicated with colored arrows.

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## **Chapter Four:**

### **Construction and characterization of capsid-display recombinant adenoviruses expressing the highly conserved M2 extracellular domain and HA2 alpha helix**

## Abstract

Influenza vaccines are critical for reducing morbidity and mortality associated with annual influenza epidemics. These vaccines protect primarily by inducing neutralizing antibodies targeting the hemagglutinin (HA) and neuraminidase (NA) proteins. Current influenza vaccines provide good protection from infection with antigenically matched virus strains but provide limited protection when circulating virus strains undergo either antigenic shift or drift [1]. The extracellular domain of the M2 protein has been proposed as a potential universal vaccine target due to its conserved nature and the ability of antibodies that recognize this domain to protect animals from influenza A virus (IAV) infection [2,3]. More recently, a highly conserved epitope of the HA protein located in the HA2 alpha helix (HA2A) has also been proposed as a universal target [4,5]. To further investigate M2 ectodomain (M2e) and HA2 based IAV vaccines, various M2e and two HA2A sequences were inserted into hypervariable regions (HVR) of the hexon capsid protein of recombinant adenovirus serotype 5 (rAd5) to display these epitopes on the surface of the virion. While immunized mice had a robust immune response against the rAd vector, antibodies against M2e or HA2A were undetectable by western blot and flow cytometry. We hypothesize that the instability of the M2e insertion into hexon prevented effective immunization against influenza *in vivo* and that the HA2A region alone is not immunogenic.

## Introduction

Influenza A virus (IAV) infections remain a major threat to public health and are responsible for approximately 500,000 deaths worldwide per year [1]. Vaccination is the most effective means of controlling morbidity and mortality associated with annual influenza epidemics. Current influenza vaccines provide protection primarily by inducing neutralizing antibodies to hemagglutinin (HA). However, an important limitation of current vaccines is that the major vaccine target is an antigenic region of the head of the HA protein and this gene is susceptible to continuous mutation. The high mutation rate allows for the selection of mutants in the human population and leads to antigenic drift [2]. Novel pandemic strains arise when a reassortment of genes occurs between animal and human influenza viruses leading to antigenic shift. Current influenza vaccines provide good protection from infection with antigenically matched virus strains but are limited in protecting against antigenic drift or shift viruses [2]. The emergence of the 2009 pandemic H1N1 is a prime example of the generation of a new strain of virus that was capable of replicating in a largely naïve population since the seasonal vaccine provided little to no protection [3]. Due to the presence of antigenic shift and drift viruses, seasonal vaccine formulations need to be evaluated on a yearly basis to match the predicted circulating strain in the upcoming influenza season. The development of a vaccine that can confer cross-protection against multiple influenza variants would eliminate a significant morbidity and mortality associated with seasonal influenza and provide more protection against pandemics.

The influenza A M2 protein is a minor integral membrane protein with a highly conserved 24 amino acid extracellular domain (M2e). It is translated from a spliced mRNA segment derived from RNA segment 7 [4]. Few M2 proteins are incorporated into virus particles but the protein is expressed at high levels on the infected cell surface and can serve as an antigenic target to the immune system [5]. Humoral immunity against M2 in a natural infection

is very weak compared to immunity against HA and NA but unlike HA and NA, there is no observed antigenic shift or drift to the M2 protein [6, 7]. Although antibodies to M2 are non-neutralizing, they are capable of protecting animals from morbidity and mortality *in vivo* [8-11].

Most HA antibodies are generated against the antigenically diverse head. However, several publications have identified HA epitopes that are conserved across multiple antigenic subtypes of HA [12-22]. Influenza A viruses can be grouped into two groups based upon their HA structure: group 1 contains H1 and H5, and group 2 contains H3 [23, 24]. The majority of IAV exhibit high degree of sequence conservation for the HA2A within a group however, there is significant sequence divergence between groups. Antibodies that recognize these conserved epitopes were capable of protecting mice from IAV infection with multiple HA subtypes of one group but not across groups [13, 15]. The primary interaction of these antibodies were localized to the alpha helix region located on the HA2 subunit (HA2A) [13, 25] and were protective by preventing the low pH induced structural changes in HA that is associated with viral-cell membrane fusion [25].

Historically, live adenovirus types 4 and 7 (Ad4 and Ad7) have been used for nearly three decades to safely protect US military personnel from serious respiratory disease. These vaccines are orally administered enteric-coated tablets containing approximately  $10^5$  TCID<sub>50</sub> of lyophilized virus which subsequently asymptotically replicates in the gut [26]. This replication induces both humoral and cell-mediated immune responses which protect against greater than 90% of subsequent Ad4- and Ad7-mediated symptomatic infections [27-30]. The innate properties of Ad that makes it such an effective vaccine can be utilized in a vaccine vector. The advantages of an Ad vector includes: natural induction of a robust cellular and humoral immune response, potent transient protein expression, efficient delivery of antigen to antigen presenting cells and easy scalable manufacturing in stable cell lines [31]. For these reasons, recombinant adenoviruses (rAd) have been used as vectors for gene therapy and for vaccination in studies using both

animals and humans [32]. Traditional rAd vectors express entire or large portions of a foreign epitope as a transgene, inserted into either early region 1 (E1), early region 3 (E3) or early region 4 (E4) of the genome [33]. However, with the exception of E3-modified vectors, most transgene expression vectors are replication-incompetent and are only capable of inducing an immune response after infection of cells by virus particles in the inoculum to produce and present antigen intracellularly. A second rAd antigen expression method exists in which shorter segments of foreign antigens are incorporated into one of the capsid proteins such that they are displayed on the surface of the virus particle. These capsid-display vectors are replication-competent and potentially immunogenic. By displaying epitopes on the capsid surface, strong humoral and cell mediated responses similar to those against Ad capsid proteins can be elicited via the exogenous pathway. It has been demonstrated that these vectors can be extremely immunogenic in mice and may offer greater efficacy in inducing humoral responses in permissive systems [34-37].

Since antibodies against M2e and HA2 have been shown to protect against influenza, replication-competent rAds were constructed that display various M2e and both group 1 and group 2 HA2A sequences in either hypervariable region (HVR) 1 or 5 of hexon. Surprisingly, despite high Ad antibody titers, recombinants that display M2e or HA2A did not induce antibody against either influenza epitope in mice. Possible explanations include virion instability, or an intrinsic lack of immunogenicity.



## Materials and methods

### *M2e and HA2A sequences*

All influenza A M2e and HA2A protein sequences were obtained from the NCBI Influenza Virus Sequence Database [38]. Sequences were aligned using ClustalW 2.0.10 [39]. The percent conservation of all IAV M2 and HA2A sequences encoding the consensus M2e or HA2A residue at each amino acid position was determined using WebLogo [40]. M2e sequences were derived from the following strains: A/WSN/33 [H1N1], A/California/4/2009, A/Swine/Best/96 [H1N1], A/Vietnam/1196/04. HA2A sequences were taken from these strains to represent group 1 and group 2 consensus sequences: A/WSN/33 and A/HK/1/68, respectively

### *Construction of recombinants*

Insertions and substitutions in hypervariable region 1 (HVR1) and hypervariable region 5 (HVR5) of hexon were made by overlap extension PCR using the mutagenesis primers listed in Table 2 and pJMG, a plasmid containing an intact wild-type (WT) adenovirus type 5 (Ad5) hexon gene [37], as a template. The three-step reaction involved an initial amplification of two different products. One was generated by using a universal left primer and a mutagenesis right primer containing M2e or HA2A sequences in the modified tail while the other product used a universal right primer and a mutagenesis left primer containing M2e or HA2A sequences in the tail. These products were purified using 1% agarose (Becton-Dickenson) and the two products were combined to generate an overlap PCR product using the universal left and right primers. The final PCR products, about 1.6kb in length, were cloned into pJMG, using *ApaI* and *SacI* sites present in the viral portions of the overlap product. WSN M2e insertions contained a novel *BamHI* restriction site for screening of pJMG colonies. Other recombinants did not contain a novel restriction site and were screened for correct PCR product size using Ad5F and HVR1 R or HVR5 R primers. All recombinant clones were verified by sequencing.

## *Cells*

Human embryonic kidney (293) cells were cultured in Essential Minimum Eagle Medium (EMEM; Lonza) containing 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco) and 2mM L-Glutamine (Gibco) at 37°C and 5% CO<sub>2</sub>. Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma) containing 10% FBS (Atlanta Biologicals), 100 U/mL penicillin (Invitrogen), 100 µg/mL streptomycin (Invitrogen), 1mM sodium pyruvate (Sigma) and 2mM Glutamax (Invitrogen) at 37°C and 5% CO<sub>2</sub>. MDCK cells stably expressing WSN M2 were cultured in identical media as WT MDCK cells supplemented with puromycin (7.5 µg/mL; Sigma) and amantadine HCl (5 µM; Sigma).

## *Calcium phosphate transfection and recombination*

Modified hexon fragments were incorporated into intact viral DNA by recombination in CaPO<sub>4</sub>-mediated transfected HEK 293 tissue culture cells [41]. pJMG plasmid DNA containing hexon modifications was digested with *PmeI* and *BamHI* and purified by ethanol precipitation. Parental viral DNA from NVDP404 (IIg) [37], an Ad5 virus containing a *Plasmodium falciparum* circumsporozoite (CSP) epitope insertion in HVR1, was digested with *NdeI* overnight. An aliquot of cut and uncut viral DNA was further digested with *EcoR5* to verify complete *NdeI*-digestion. NVDP404 DNA digested with *NdeI* was precipitated with ethanol. Digested plasmid (2-5µg) and viral DNA (2-5µg) was supplemented with salmon sperm DNA (10-16µg) for a total of 20µg of DNA and added to Hepes-buffered saline pH 7.5 (HBS) [42] for a final volume of 950µL. 50µL of 2.5M CaCl<sub>2</sub> was rapidly added to the DNA/HBS mixture and incubated for 20-30 minutes before being added drop wise to two plates of HEK293 cells. Media was replaced five hours later with fresh EMEM and cells were maintained in culture until approximately 50% of cells showed cytopathic effects (CPE). Whole cell lysates were collected and analyzed by

western blot for recombination followed by three cycles of freeze/thaw to release cell-associated virus. Viruses were declumped by the addition of NP40 or IPEGAL (Sigma) for a final concentration of 0.05% and 1/10 volume of 1,1,2-Trichlorotrifluoroethane (Sigma). The organic mixture was mixed vigorously for 2 minutes and spun at 2500-5000 RPM for five minutes for phase separation. Supernatant containing declumped viruses were used for plaque purification on HEK 293 cells. Plaque-picked viruses were screened for purity and insertion of the antigen by western blot and sequencing. Desired viruses were grown to high titer in 10-20 14cm tissue culture dishes of HEK 293 cells. Upon showing CPE in 50% of the cells, whole cell lysates were collected and viruses were purified using CsCl.

#### *CsCl virus purification*

rAd infected cells were collected and centrifuged for 10 minutes at 6,000 rpm in a Sorvall GSA rotor. Cell pellets were resuspended in 25 mL of supernatant per 200-400 mL of original culture. HBS pH 7.4 (Gibco) was added to resuspended cells for a final concentration of 20mM. Concentrated cell suspensions were freeze-thawed three times or cell-associated virus was extracted using the declumping method described previously. Freeze-thawing yielded in the highest plaque forming unit (pfu)/particle ratio while extraction using organic solvents resulted in the best particle yield. The method for release of cell-associated virus depended on whether the virus was to be used for infections where a high pfu/particle ratio is desired or as an immunogen or for DNA extraction when yield is most important. Large debris from the lysates were removed using a clinical centrifuge at 2,500 rpm for 5 minutes. The supernatant was centrifuged again at 15,000 rpm using a Sorvall SS34 or SA600 rotor for 10 minutes to remove more cell debris. Clarified lysate was layered over a discontinuous CsCl gradient containing 4 mL CsCl density 1.25g/mL and 5mL CsCl density 1.70g/mL in a 35 mL centrifuge tube (Sorvall). Gradients were centrifuged for 90 minutes at 17,000 rpm using the Sorvall SV288 vertical rotor. The lowest and dominant band at the interface of the two CsCl solutions was collected drop-wise and centrifuged

in a continuous CsCl gradient of density 1.34g/mL for at least 16 hours at 35,000 rpm in a Sorvall TV865 rotor. The dominant band was collected and recovered virus was dialyzed twice in 500mL of storage solution containing 0.15M NaCl, 0.5mM Ca<sup>+2</sup>, 0.9mM Mg<sup>+2</sup>, 5% sucrose and 20mM Hepes pH 7.4 at 4°C. Particle number in gradient-purified virus preparations was determined from the A<sub>260</sub> of SDS-disrupted particles using the formula A<sub>260</sub> x dilution factor x 1.1x10<sup>12</sup> particles/mL [43].

#### *SDS-PAGE and western blots*

Infected cells were lysed in 1% SDS (Fisher Scientific) in PBS and lysates or purified virions were mixed 1:1 in 2x SDS-PAGE sample buffer [44]. Samples were resolved in a 10% poly acrylamide gel and either transferred to polyvinylidene difluoride membranes (PVDF-FL; Millipore) or used for Coomassie or silver stain (Bio-Rad) to view total protein. For immunoblots, wash buffer contained PBS with 0.3% Tween 20 (Sigma) and the blocking buffer was wash buffer with 5% nonfat dry milk. Membranes were blocked for 30 minutes at room temperature (RT), incubated for 2 hrs at RT with primary antibody, washed three times for 5 min each, incubated for 1 hr at RT with secondary antibody and washed four times 5 min each. Primary and secondary antibodies were diluted in blocking buffer. Primary antibodies used were mouse anti-M2 14C2 monoclonal antibody (1:500 dilution), rabbit anti-late adenovirus late protein antisera (1:3000 dilution), mouse anti-CSP 2A10 antisera (1:3000) and V-314-511-157, a polyclonal goat anti-HA0 A/PR/8/34 antibody (1:500 dilution; National Institute of Allergy and Infectious Diseases). The Alexa Fluor 647-conjugated secondary antibodies used were a goat anti-mouse immunoglobulin (IgG) and a goat anti-rabbit IgG (all at a 1:500 dilution; Invitrogen). For visualization, membranes were scanned using an FLA-5000 phosphoimager (Fujifilm).

#### *Diagnostic PCR and sequencing of pure virus particles*

All recombinant viruses to be used for PCR or for sequencing needed to undergo a proteinase K (Roche) treatment to disrupt the capsid in order to access the DNA [45]. Pure virus ( $\sim 10^7$  particles/mL) or plaque picks (50 $\mu$ L) were incubated with 5 $\mu$ g of proteinase K for 30 min at 37°C. Proteinase K was inactivated by a 20 min incubation at 95°C. A portion of this treated sample was either sent away for sequencing or used for PCR. Recombinant viruses expressing HA2A were screened by diagnostic PCR using Ad5 F and either WSN HA2A R or HK68 HA2A R (Table 2) depending on the sample. Products were run on an agarose gel and visualized with ethidium bromide staining.

#### *Plaque purification/assay*

Plaque assays were carried out by infecting confluent 60mm tissue culture petri dishes (Nunc) of 293 cells with the indicated deplumped viruses serially diluted in medium. Cells were infected with 1mL of diluted virus and incubated for 2 hrs at 37°C, occasionally rocking the dishes to distribute the inoculum evenly. Inocula were aspirated and cells were overlaid with 5mL of 0.9% Bacto-agar (Difco) and 2% FBS in MEM (Gibco) without phenol red, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin (Gibco). After overlays had solidified, cells were incubated at 37°C and were fed with an additional 2.5mL of fresh agar medium every three days. On the sixth day, 2.5mL agar medium was added containing neutral red (Sigma) at a 1:40 dilution for visualization of plaques. For plaque purification of viruses, individual plaques were picked using a glass Pasteur pipette and frozen in 0.5mL EMEM media to be analyzed at a later time. For plaque assay, plaques were counted until no new plaques arose.

#### *Mouse immunization and serum sample collection*

Inbred 4- to 8- week old female BALB/c mice (NCI) in groups of five or 10 were immunized subcutaneously three times at three week intervals with a fourth immunization 12 weeks later in one experiment. Mice were immunized with  $10^{10}$  particles of human papilloma

virus (HPV) L2 recombinants (Q or 2.6.1), WSN M2e or WSN HA2A (Table 3). For immunizations supplemented with adjuvant, 50 µg/mouse Alum (Al(OH)<sub>3</sub>) and 5 µg/mouse MPL were used. Viruses were mixed with adjuvant overnight to ensure that adjuvants did not fall out of solution and mice were immunized as above. Blood was collected in serum separator tubes (Microtainer) from each mouse by tail bleeds three weeks after each immunization and prior to the subsequent immunization. Tubes were spun in a microcentrifuge (Denville Scientific) at 5000 rpm for 10 min to separate the sera from cells and clotting factors. Sera were collected and stored at -80°C in microcentrifuge tubes.

#### *Ad5 and L2 ELISA*

ELISA plates for Ad5 (Immulon 2HB; Thermo Scientific) and L2 (MaxiSorp; Thermo Scientific) were coated with either 1µg of Ad5 or 100ng of L2 protein per well in 100µL and incubated at 4°C overnight. Ad5 plates were washed three times with PBS containing 0.1% Tween 20 (PBS-T) followed by three washes with PBS. Both Ad5 and L2 plates were blocked with 1% BSA in PBS (PBS-BSA) for 1 hr at RT followed by dilutions of sera in PBS-BSA starting at 1:500 (five-fold serial dilutions) for Ad5 or at 1:50 (two-fold serial dilutions) for L2 for 1 hr at RT. Plates were washed as described previously and incubated with secondary horseradish peroxidase-conjugated anti-mouse IgG (1:5,000 dilution; GE Healthcare) or anti-monkey Ig (1:1000 dilution, Nordic) at RT. Plates were washed a final time and developed for 15 min with ABTS Peroxidase Substrate System (KPL). The developing reaction was stopped with 1% SDS and optical density at 405nm was measured using a Molecular Devices Emax microplate reader. Experiments were performed in triplicate and the endpoint ELISA titer was determined as the highest dilution at which the optical density was two times greater than background (wells containing sera but no antigen). Positive controls was human sera for Ad5 and RG-1, a monoclonal antibody recognizing an HPV16 L2 epitope [46]. All L2 ELISAs were performed by Tanwee Alkutkar.

### *Influenza virus infection*

Confluent MDCK cells grown in 6-well plates were washed twice with PBS with calcium and magnesium (PBS+; Invitrogen) to remove FBS. MDCK cells were infected with rWSN, a recombinant virus of A/WSN/33 [47], at a multiplicity of infection (MOI) of 5 50% tissue culture infectious dose (TCID<sub>50</sub>) per cell in 250µL infection media (DMEM supplemented with 0.5% BSA, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, 2 mM Glutamax and 4 µg/mL *N*-acetyl trypsin (NAT; Sigma)) at RT with rocking for 1 hr. Cells were then washed twice with PBS+ and incubated with 1 mL infection media at 37°C for 9 hrs. Media was aspirated off, wells were washed two times with PBS and incubated at 37°C in 0.5 mL of 2x trypsin to remove cells. Cells were then processed for use in western blotting or flow cytometry.

### *Flow cytometry*

Cells were removed from the tissue culture plate by trypsinization and fixed by 2% paraformaldehyde (Sigma) in PBS for 15 min at RT. Cells were spun down at 1200 rpm for 3 min and washed 2x with PBS. Samples were stored at 4°C in 1mL PBS overnight. Cells were resuspended in blocking buffer containing 0.5% bovine serum albumin (BSA; Sigma) and 3% normal goat serum (NGS) in PBS for 45 min on ice, incubated for 1hr in primary antibody or mouse sera on ice, washed three times with PBS, incubated for 1 hr on ice in secondary antibody, washed three times with PBS and fixed with 2% paraformaldehyde for 15 min at RT. Cells were washed twice with PBS and transferred to Falcon 5mL polystyrene round-bottom tubes (Becton-Dickenson) for analysis. All mouse sera, primary and secondary antibodies were diluted in blocking buffer. Monoclonal primary antibody 14C2 and goat anti-mouse Alexa Fluor A647-conjugated secondary were used at 1:500. The cells were analyzed by flow cytometry (Becton Dickinson FACS Calibur) by using FlowJo software.

### *Influenza challenge of Balb/c mice*

Ten 9 month old female Balb/c mice previously immunized 3x with either rAd WSN HA2A or 2.6.1 supplemented with Alum and MPL adjuvants were challenged. Mice were anesthetized with 100  $\mu$ L of a ketamine (100mg/kg of body weight) – xylazine (20mg/kg) cocktail administered via intraperitoneal injection. Mice received a 20  $\mu$ L intranasal inoculation of 10 LD<sub>50</sub> of rWSN virus diluted in DMEM with penicillin-streptomycin and 2  $\mu$ g/mL *N*-acetyl trypsin (Sigma). 1LD<sub>50</sub> of rWSN was previously determined to be  $5.8 \times 10^3$  PFU/mL [48]. Animals were monitored for 16 days postinfection for morbidity and mortality. Mice were weighed daily and the data were graphed as the percent of weight at the time of infection.



## Results

### *Construction of rAd expressing conserved influenza A epitopes*

Current seasonal influenza vaccines and natural infection with influenza A viruses primarily induce antibodies to the immunodominant HA head and NA proteins. However, due to the selective pressure imposed by the immune system, these epitopes evolve rapidly, resulting in antigenic drift and the ability of different viral strains to evade the immune response. Although these epitopes have high variability, there are other epitopes within HA and other IAV proteins that are much more conserved. However antibodies are not readily raised against these epitopes by seasonal vaccines or natural infection. These include the HA2A located in the stem of the HA molecule and the M2e which is expressed at high levels on the surface of infected cells (Figure 1). Antibodies specific for either the M2e or HA2A epitopes have been shown to be protective *in vivo* albeit by different mechanisms. HA2A antibodies are capable of neutralizing infection by preventing the low pH structural changes in HA necessary for viral fusion with the endosomal membrane [25]. Antibodies targeting the M2e, while not neutralizing, are capable of protecting mice from a lethal challenge *in vivo*, potentially by antibody-dependent cell cytotoxicity (ADCC) [8, 49].

While each of these epitopes are more conserved than the diverse antigenic HA head and NA proteins, there is still some variation between viral strains. M2e is highly conserved within viruses from a particular animal species making this an attractive vaccine target for a ‘universal’ influenza vaccine (Figure 1A ). However, it has been shown that monoclonal antibodies raised against human IAV M2e do not recognize the M2e of swine, avian and the 2009 H1N1 influenza viruses due to amino acid changes in the epitope region, which spans amino acid 6-15 (Figure 1A and 1B). Monoclonal antibodies have been raised against the more highly conserved N-terminal region of the M2 protein [9], amino acids 1-8, and these were capable of recognizing M2e from

multiple animal species including an H5N1 and the 2009 pandemic H1N1 (pH1N1) (Figure 1A and 1B). To create an M2e-based ‘universal’ vaccine that would protect from seasonal and pandemic influenza A viruses, it would be necessary to vaccinate with each M2e sequence specific for human, swine, avian and the 2009 pH1N1.

Due to the many different strains of IAV, HA molecules can be grouped in either group 1 or group 2 depending on their structure [23, 24]. The majority of IAV exhibit high degree of sequence conservation for the HA2A helix within a group however, there is significant sequence divergence between groups (Figure 1C-E). Antibodies targeting this region exhibit little to no cross-reactivity to HA molecules within a different group [13, 15]. Thus to protect against all IAV, both group 1 and group 2 HA2A sequences would need to be utilized in a vaccine.

To make a prototype ‘universal’ vaccine, the influenza M2e and HA2A sequences were inserted into either HVR1 or HVR5 of rAd of serotype 5 (Ad5) by four-primer PCR (Table 1 and Table 2). Two independent PCR reactions took place, one with a universal left primer (Ad5F) and a mutagenesis right primer containing either M2e or HA2A sequences. The other reaction contained a universal right primer (Ad5R) and a mutagenesis left primer containing either M2e or HA2A sequences (Table 2). The mutagenesis primers overlapped by approximately 20 base pairs in order to facilitate the fusion of the two mutagenesis products. These products were purified and used in the subsequent reaction as the template with the Ad5F and Ad5R universal primers to create an overlap PCR product of approximately 1.6 kb in length. The final product was verified by screening for a novel restriction enzyme site, in the case of rAd expressing WSN M2e (rAd WSN M2e), or diagnostic PCR primers in the case of the HA2A constructs and sequencing.

The modified hexon fragments containing the IAV epitopes were incorporated into intact Ad5 viral DNA by recombination in 293 cells through calcium-phosphate mediated transfection [41]. Briefly, the pJMG plasmid containing the modified hexon fragments and the parental viral

DNA from NVDP404, an Ad5 virus containing a *Plasmodium falciparum* CSP epitope insertion in HVR1 [37], were restriction digested overnight and ethanol precipitated. Digested plasmid DNA and parental viral DNA were transfected into 293 cells. Recombinants from the transfection reconstitutes an intact viral genome incorporating the IAV epitope and whole cell lysates were collected when approximately 50% of the cells showed signs of CPE.

Viruses from the whole cell lysates were plaque-purified to isolate a single virus clone. These plaque picks were screened for the presence of recombinant viral DNA containing M2e or HA2A sequences by western blot and diagnostic PCR (Figure 2). To confirm the presence of Ad structural proteins in the plaque pick, blots were probed with a polyclonal anti-late antibody that detects hexon, penton, protein IIIa, V and VI. WT Ad5 was used as a control for the size and correct banding pattern of each of the structural proteins. Plaques from the rAd WSN M2e, rAd WSN HA2A and rAd HK68 HA2A recombination showed the correct banding pattern and approximate size for the Ad structural proteins, indicating that Ad was present in the samples (Figure 2A and D).

The parental viral DNA in the recombination events expressed a *P. falciparum* CSP epitope [37] and a successful recombination event would result in viruses lacking this epitope. To determine the presence of the CSP epitope, blots were probed with 2A10, a monoclonal antibody recognizing the repeat region of CSP. While the parental NVDP404 virus and *P. berghei* sporozoites expressing the *P. falciparum* CSP 2A10 epitope showed the presence of the CSP epitope, the rAd expressing WSN M2e, WSN HA2A or HK68 HA2A were negative, demonstrating that the plaque picks were devoid of parental virus and solely contained virus lacking the CSP epitope (Figure 2B and E).

In order to demonstrate that the plaque picks contained recombinant virus expressing the M2e epitope, blots were probed with 14C2, a monoclonal M2e antibody. Only the rAd WSN

M2e virus plaque pick reacted with the 14C2 antibody, indicating that this virus contained the M2e epitope. Antibodies recognizing the HA2A sequence do not currently exist, so to determine if plaque picks contained the HA2A epitope, diagnostic PCR primers were used, with the sequenced plasmid containing WSN or HK68 HA2A as a positive control. Both WT Ad5 and NVDP404 were negative for HA2A while the pJMG HA2A plasmids, rAd WSN HA2A and HK68 HA2A plaque picks contained the HA2A sequence (Figure 2F). The plaque picks containing the correct epitope or sequence for rAds WSN M2e, WSN HA2A and HK68 HA2A were confirmed by sequencing.

#### *Analysis of purified rAd particles expressing M2e or HA2A*

Multiple plaque picks from rAd WSN M2e were associated with products of multiple sizes that contained the M2e epitope, as demonstrated by its recognition by 14C2 (Figure 2C). We hypothesize that these represent cleavage products of hexon where the M2e epitope was inserted. Several sizes of these M2e cleavage products correlated with anti-late reactive bands that were only present in the rAd WSN M2e plaque picks and were not of the correct size of any known Ad structural protein (Figure 2A). To determine if such products were being produced in the rAd HA2A viruses and if any of the rAds were incorporating such products into the virion we examined purified virus particles. Plaque picks from rAd WSN M2e, WSN HA2A and HK68 HA2A were used to infect 293 cells and virus was purified by CsCl gradients. Purified virus was loaded onto an SDS-PAGE gel and analyzed either by silver stain (Figure 3A) or Coomassie stain (Figure 3B) in order to view the total protein incorporated into the virus particles. The banding pattern for WT Ad5, rAd WSN HA2A and rAd HK68 HA2A were similar and the rAd HA2A viruses did not appear to contain unexpected proteins in its virions in substantial amounts (Figure 3A and B). In contrast, rAd WSN M2e virions contained multiple unknown proteins that were of fairly dark intensity, indicating a high concentration of those proteins within the virion. Interestingly, two of the proteins, one slightly smaller than hexon and another slightly smaller

than penton but larger than IIIa, correlated to the sizes of cleavage products seen in the plaque picks that were reactive with 14C2 (Figure 2C) suggesting that some of the unknown proteins incorporated into the rAd WSN M2e virions are hexon-M2e cleavage products.

To assess the overall impact that the HVR insertion had on viral growth characteristics, WT Ad5, rAd WSN M2e and WSN HA2A were plaqued to determine the average particle to PFU ratio. This ratio is indicative of how infectious these viruses are and whether the purified virus contains a large proportion of non-infectious particles. WT Ad5 had an average particle:PFU ratio of 70 which was similar to rAd WSN HA2A, with a particle:PFU ratio of 64.7. In contrast, rAd WSN M2e had a particle:PFU ratio of 188, more than 2x that of WT Ad5, indicating a larger amount of non-infectious particles. While the insertion of the WSN HA2A sequence into HVR5 did not appear to have any significant adverse effects on virus, the insertion of the WSN M2e sequence into HVR1 impacted the infectiousness of the virus, most likely due to the incorporated cleavage products.

#### *Protein production and growth kinetics of rAd WSN M2e*

The high particle:PFU ratio and presence of potential cleavage products into the virion suggests that the rAd WSN M2e virus is not stable. It also grew to lower titer and during CsCl purification, a substantial fraction of virus was lost during the equilibrium centrifugation, leading us to further suspect instability of the virion. To investigate this observation further, 293 cells were infected with WT Ad5 or rAd WSN M2e at an MOI of 5 PFU. Cell lysates were collected at various time points and were analyzed by western blot for the production of Ad structural proteins (Figure 4A) and hexon-M2e cleavage products (Figure 4B). Hexon was detected as early as 16 hours post infection (hpi) in both viruses and the production of Ad capsid proteins was similar until 32hpi when it was apparent that cells infected with WT Ad5 had more capsid proteins than those infected by rAd WSN M2e (Figure 4A). At this same time point, cleavage

products of hexon-M2e were apparent in the rAd WSN M2e virus (Figure 4B). The presence of hexon-M2e cleavage products steadily increased alongside the increase in capsid proteins, indicating that as more hexon proteins were produced, more cleavage products were produced as well and accumulated within the cell, suggesting instability of the hexon protein.

Lysates from this growth curve were also plaqued to determine the amount of infectious virus in each sample. By 16hpi, there was a high titer of infectious WT Ad5 virus that continued to increase until 48hpi. The rAd WSN M2e displayed similar growth kinetics however, it was always at least 1 log lower in infectious virus formation, demonstrating a delay in infectious virus production.

The insertion of the WSN M2e sequence into HVR1 of rAd5 did have an impact on the overall viral fitness, with the production of an unstable hexon and delay in infectious virus production. However, virus was still able to productively infect cells, albeit at a lower rate than WT Ad5. Although the rAd WSN M2e virus was more unstable than WT Ad5, it was still possible that the virus could act as an efficient immunogen for a vaccine.

#### *Immunogenicity of rAd WSN M2e*

The immunogenicity of the rAd WSN M2e virus was investigated in female Balb/c mice. Ten 4-8 week old mice received three subcutaneous (subQ) injections at 3 week intervals of  $1 \times 10^{10}$  particles of recombinant virus (Figure 5A). A group of 5 control mice were injected similarly with an rAd expressing the human papilloma virus serotype 16 (HPV16) L2 [46] epitope in HVR1 (Q virus; Table 3). Individual sera was collected every three weeks, prior to viral boost, and was analyzed by ELISA for Ad5 antibodies. All rAd WSN M2e mice developed a robust immune response to Ad5 and reached similar peak titers after the 3<sup>rd</sup> immunization (Figure 5B). Although Q mice were immunized similarly, their Ad5 antibody titers were much lower. Pooled sera from the 3<sup>rd</sup> bleed was used to probe Western blots of MDCK cells stably expressing

M2 to detect M2 antibodies. M2 was visible around 14kD when probed with 14C2, however pooled sera from rAd WSN M2e immunized mice was unable to detect the protein indicating the absence of an antibody response against a linear M2e epitope (Figure 5C). Although there is a crystal structure for the M2 transmembrane and C terminal domains [50-52], the structure of the M2e is unknown. To determine if M2e antibodies were induced against a conformational epitope, immunostaining for M2 with 14C2 or pooled sera from rAd WSN M2e or Q virus was performed by flow cytometry of M2 expressing and non-expressing MDCK cells. While 14C2 was able to recognize M2 on the surface of MDCK cells, pooled sera from rAd WSN M2e and Q mice was unable to detect any M2. These results indicate that although the vaccinations were successful, with the mice developing an immune response against the rAd capsid, M2e-specific antibodies, recognizing either a linear or conformational epitope, were not detectable.

#### *Immunogenicity of rAd WSN HA2A*

To determine the immunogenicity of the rAd WSN HA2A virus, in collaboration with Dr. Richard Roden and Tanwee Alkutkar, 5 Balb/c female mice were injected subQ with  $1 \times 10^{10}$  particles of either rAd WSN HA2A or 2.6.1, an rAd expressing HPV16 L2 antigen in HVR5 (Table 3) [46]. Mice were vaccinated every 3 weeks and antibody titers against Ad5 were monitored by ELISA (Figure 6A). Both rAd WSN HA2A and 2.6.1 mice developed equivalent peak antibody titers after the 2<sup>nd</sup> immunization (Figure 6B). To demonstrate that an immune response could be induced against a foreign antigen inserted into the HVR5 region, L2 antibodies were measured by ELISA. Although the response was variable, all mice did develop a detectable immune response against the L2 antigen (Figure 6B). Since the immune response to the L2 antigen was low, mice received a 4th immunization 20 weeks after priming. Pooled sera was used to probe western blots of infected cell lysates for the presence of HA2A antibodies. While the pooled rAd WSN HA2A sera does react with several bands at the size of HA0, these bands are also present in uninfected cell lysates and also reacts with the 2.6.1 pooled sera, indicating

that these bands are due to nonspecific antibody binding. More convincingly, rAd WSN HA2A sera was unable to react with HA2, which contains the HA2A epitope and is recognized by the anti-HA0 PR8 sera, indicating that there are no detectable antibodies against the HA2A region (Figure 6C). To determine if conformation specific antibodies against HA2A were induced, immunostaining by flow cytometry of rWSN infected cells HA2A with pooled sera from rAd WSN HA2A was conducted. However, conformational antibodies against HA2A were not detected by flow cytometry of infected cell lysates (Figure 6D). M2 was readily detected by 14C2 confirming that cells were infected with rWSN.

L2 is known to be an immunogenic epitope [53, 54] however the immune response against it was variable and low in comparison to the antibody response against Ad5. Little is known about the immunogenicity of the HA2A region. In order to increase the immune response against L2 and potentially be able to detect antibodies against HA2A, vaccinations were supplemented with monophosphoryl lipid A (MPL) and aluminum salts (alum) adjuvants. Both of these adjuvants induce an immune response by different mechanisms. Alum has a depot effect to ensure a long lasting immune response that is induced by activation of the NLRP3 inflammasome and release of DNA from dying cells which mediates a strong TH2 adjuvant effect while MPL activates antigen presenting cells by interaction with TLR2 and TLR4 leading to a Th1 immune response [55, 56]. It has been shown that the combination of these adjuvants can successfully prime long-lived memory CD8<sup>+</sup> T cells and protect mice from a lethal influenza A challenge [57].

10 Balb/c female mice were immunized similarly with  $1 \times 10^{10}$  particles of rAd WSN HA2A or 2.6.1 every 3 weeks but immunizations were supplemented with MPL and alum adjuvants (Figure 7A). The Ad5 antibody titers increased in all the mice, along with the L2 antibody titers for the 2.6.1 mice (Figure 7B). Despite the drastic increase in the anti-L2 immune response and the increase in the anti-Ad5 immune response, there was still no detectable



antibodies to HA2A by western (Figure 7C) or flow cytometry (Fig 7D) of infected cells. Since an antibody response was generated against an immunogenic epitope, L2, inserted into the same location of the hexon protein as HA2A, the lack of an immune response against HA2A suggests that its insertion in this region is not sufficient to induce a detectable humoral immune response.

While mouse sera did not test positive for the presence of anti-HA2A antibodies, it is possible that antibodies to this region were induced but not detectable or that immunization stimulated a T cell response. To test if mice immunized with rAd WSN HA2A exhibited any protection from homologous influenza challenge, both adjuvant-supplemented rAd WSN HA2A and 2.6.1 immunized mice were challenged with 10 LD<sub>50</sub> of rWSN. All mice initially exhibited weight loss however, there was no statistical difference between rAd WSN HA2A and 2.6.1 vaccinated mice, suggesting that rAd WSN HA2A did not induce a humoral or cellular immune response that was protective (Figure 8). While this challenge used 10 LD<sub>50</sub>, both groups recovered from the challenge. This indicates that while mice did become productively infected, as depicted by the initial morbidity, it was not a lethal challenge. Diluted virus stock used to infect the mice was backtitered and it was determined that the mice received the correct dose. Thus, it is likely that the age of the mice was a significant factor in the challenge as the number of PFU of rWSN that constituted 1 LD<sub>50</sub> was based on experiments with 8-12 week old female mice whereas mice used in this experiment were approximately 9 months old.

## Discussion

With the emergence of the 2009 H1N1 and outbreaks of avian H5N1 in countries around the world, there has been resurgence in the development of a ‘universal’ vaccine to protect against seasonal and pandemic IAV. With current vaccination strategies, most antibodies are induced against the immunodominant head of the HA protein. However, due to the variable nature of this region, there is a lack of cross-protection between viruses that do not closely correspond to the vaccine strain. Current efforts towards a universal vaccine have mainly focused on the development of antibodies against conserved regions of the virus. The 24 amino acid sequence of the M2e is highly conserved and antibodies against this region are protective *in vivo* but are not induced in natural infection. Studies with a ‘headless HA’ construct in which the immunodominant globular domain was removed from the full-length HA has demonstrated the ability to induce more broadly protective antibodies towards the more highly conserved stem region of HA [19, 58].

Our strategy for creation of a ‘universal’ influenza A vaccine was based upon the exemplary safety and success of the oral US military vaccine against respiratory disease caused by Ad4 and Ad7 [26], along with the knowledge that an immune response can be elicited against foreign epitopes inserted into capsid proteins of Ad. We inserted the influenza A M2e epitope and HA2A region into either HVR1 or HVR5 of the hexon protein (Table 1), creating a total of 6 different rAd. The hypothesis was that while the M2e by itself is poorly immunogenic and the immunogenicity of HA2A was unknown, the insertion of these epitopes into rAd would enhance immunogenicity by acting as a natural adjuvant and an immune response, similar to that against Ad capsid proteins, would be developed against the foreign epitopes. Two rAds, rAd WSN M2e and rAd WSN HA2A, were successfully constructed and characterized *in vitro*.

The human WSN M2e was inserted into HVR1 of hexon and was readily recognized by a monoclonal antibody, 14C2, to the M2e (Figure 2). However, the 14C2 antibody also recognized smaller products that reacted with a polyclonal antibody to the Ad capsid proteins. This suggests that the insertion of the M2e sequence into HVR1 resulted in virion instability specifically within and around the insertion, leading to cleavage products that contained hexon and M2e sequences. These products were incorporated into the virus particle and appeared to accumulate over time in the viral life cycle with their presence being evident immediately upon hexon translation (Figure 4A). Although the M2e-containing virions appeared to be unstable and the virions were extremely difficult to purify by conventional cesium chloride methods, the insertion only had a small affect on viral replication as the rAd WSN M2e virus was still capable of multiple rounds of replication (Figure 4B). Given that the virus was stable enough to replicate *in vitro*, it was tested *in vivo* as a vaccine. Protective M2e antibodies have been induced in a variety of ways including injection of the full length protein with adjuvant [59], DNA administration [60], fusion to carrier proteins such as hepatitis B core protein [61, 62], keyhole limpet hemocyanin [63, 64], flagellin [65], incorporation into liposomes [66] and various viral vectors [67-71]. Thus it was hypothesized that a robust humoral immune response would be induced against M2e inserted into HVR1. While the vaccine did stimulate antibodies against the vector, there were no detectable M2e antibodies present.

Subsequently, Zhou *et al* published a study where the M2e was inserted into hexon variable region 1 (VR1) and VR4 of chimpanzee-derived replication-defective Ad vector, AdC68 [71]. This vector also expressed a transgene of three copies of the M2e sequence fused to the IAV nucleoprotein (NP) gene. Insertion of the epitope into VR1 or VR4 did not alter vector fitness as these hexon-modified vectors rescued easily and had similar growth characteristics to WT AdC68. In mice, the construct inserted into VR1 induced a robust antibody response to M2e and T cell response against NP. Moreover, it protected 80% of inbred and 100% of outbred mice

from a lethal challenge of influenza [71]. The results of this study contrast with the inability of our rAd WSN M2e to induce an immune response. This is most likely due to the instability of the rAd WSN M2e construct. It is unclear why our vector was unstable. While it could be due to insertion of a foreign epitope into HVR1, we have successfully inserted epitopes into this region previously [37]. Additionally, all tested rescued clones of rAd WSN M2e demonstrated the same issue suggesting that this was not due to an artifact of a particular clone and we were unable to plaque-purify rAd containing avian, swine or pH1N1 M2e sequences inserted into HVR1 or HVR5, most likely due to similar instability. It is also possible that there is some innate property of the M2e sequence that induces cleavage or degradation however, the same sequence was successfully inserted into AdC68, suggesting that the instability may be a combination of the M2e insertion into Ad5. Inserting the M2e into HVRs of a different Ad serotypes could prevent breakdown and allow for an immune response to be induced *in vivo*. However, it would be interesting to insert M2e into other rAd capsid proteins such as pIX and fiber to test if the construct is more stable and increase its immunogenicity.

Several publications have described the isolation and characterization of broadly protective HA antibodies that recognize epitopes conserved across multiple antigenic subtypes [13, 15]. These antibodies recognized a conformational epitope, binding to residues on HA1 and HA2. Most of the interacting surface between the antibody and its epitope was localized to the HA2A helix, suggesting that the HA2A helix alone could serve as a rationally designed antigen. The HA2A helix is conserved within the phylogenetic group 1 HA but not subtypes of group 2 as there is significant sequence divergence across groups and antibodies recognizing this region are only cross-protective within subtypes of the same group. A ‘universal’ vaccine based upon HA2A would have to consist of both WSN (group 1) and HK68 (group 2) HA2A sequences. Both the WSN and HK68 HA2A sequences were inserted into HVR5 and successfully rescued (Figure 2). Neither of these viruses exhibited any of the similar instability issues associated with the rAd

WSN M2e construct as they grew to high titer and did not incorporate any detectable cleavage products (Figure 3) however, *in vivo* experiments were conducted with only the rAd WSN HA2A. Our hypothesis was that the rAd WSN HA2A virus would induce antibodies in mice against the HA2A region that would be protective against a lethal rWSN but not an HK68 challenge due to amino acid differences in the sequences. As a positive control for the ability to induce an immune response against an inserted epitope into HVR5, we collaborated with Dr. Richard Roden and immunized additional mice with 2.6.1, an rAd expressing the HPV16 L2 epitope in HVR5 (Table 3). Despite the ability to induce antibodies against L2 and rAd, there were no detectable antibodies by western blot or flow cytometry against HA2A (Figure 6). The addition of MPL and alum adjuvants to the vaccination regimen increased the humoral response against L2 and Ad5 but was not able to induce antibodies to HA2A (Figure 7) and mice were not significantly protected from challenge with rWSN as compared to the 2.6.1 immunized controls. This data suggests that, while antibodies can be naturally induced against the HA2 stem region, making primary contacts with HA2A, this region alone is not immunogenic and fails to induce a detectable immune response even in the presence of adjuvants.

Our results describing the inability to induce a humoral response against HA2A is in agreement with Schneemann *et al* where they inserted the HA2A helix from the human 1918 H1N1 pandemic virus into loops of the Flock House Virus (FHV) capsid [72]. It is important to note that the 1918 H1N1 HA2A sequence that was inserted into FHV is identical to the WSN HA2A sequence used in our study. To preserve the helical conformation of HA2A, the helix was inserted into a B2 scaffold, a small helix-turn-helix nonstructural protein encoded by FHV, with respect to the B2 helix turns in order to maximize solvent exposure of residues that make contacts with previously characterized HA2 antibodies, such as CR6261 [15]. While antibodies were induced against FHV and the B2 scaffold, a very weak humoral immune response was induced in only two out of four mice and with only one B2/HA2A construct. Antibodies to HA2A that were

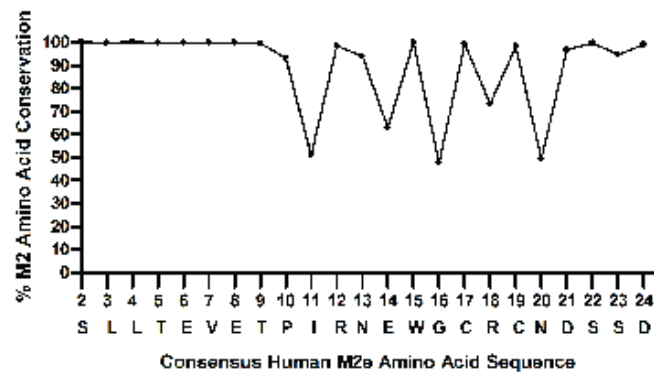
induced did react with multiple HA subtypes of the same group however these were insufficient to protect mice from a lethal PR8 challenge which belongs to group 1 HA in addition to the 1918 H1N1. One amino acid difference exists in the HA2A sequence between 1918 H1N1 sequence and the challenge PR8 virus thus a new B2/HA2A construct was made that displayed the HA2A sequence of PR8. Despite the addition of adjuvant to the vaccination regimen, only 25-37.5% of mice created antibodies to HA2A and sera from these mice were unable to neutralize PR8 *in vitro* [72]. While it is possible that *in vitro* and *in vivo* neutralization did not occur because of insufficient antibody titers, low avidity or affinity antibodies, or potential steric restrictions preventing neutralization, given our data, it is likely that HA2A alone is not sufficient as an antigen to induce broadly neutralizing antibodies.

We have demonstrated that, even though capsid display rAd approaches have worked in the past, it did not induce an immune response against two highly conserved epitopes of influenza. However, these studies were conducted in a mouse model where Ad does not replicate. It is possible that in a productive host such as monkeys or humans, the replication of the rAd IAV constructs would facilitate antibody production and induce a T cell response against the IAV epitopes. These epitopes could also be presented in the context of other Ad capsid proteins. Insertions could be made into the amino terminus of fiber or into the carboxy terminus of pIX. Several studies have suggested that insertions into the HI loop of the fiber knob are better detected in native virions and are more immunogenic [73, 74]. Alternatively, other conserved IAV epitopes could be utilized to develop a ‘universal vaccine’, such as the HA2 long alpha-helix [22], the HA fusion peptide [75] or uncleaved HA0 [76, 77]. These epitopes and rAd capsid insertion sites remain to be investigated for their use in a ‘universal’ influenza A vaccine.

A.

M,e	1	5	10	15	20	25																			
WSN H1N1	M	S	L	L	T	E	V	E	T	P	I	R	N	E	W	G	C	R	C	N	D	S	S	D	P
2009 pH1N1	M	S	L	L	T	E	V	E	T	P	T	R	S	E	W	E	C	R	C	S	D	S	S	D	P
Swine H1N1	M	S	L	L	T	E	V	E	T	P	T	R	N	G	W	E	C	R	Y	S	D	S	N	D	P
Avian H5N1	M	S	L	L	T	E	V	E	T	P	T	R	N	E	W	E	C	R	C	S	D	S	S	D	P

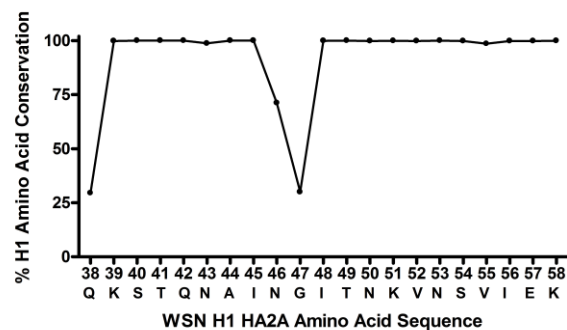
B.



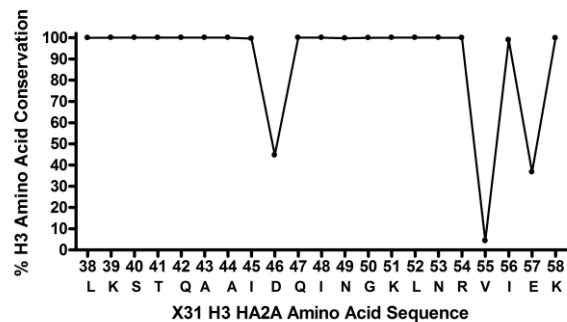
C.

HA2 A Helix	40					45					50					55					
WSN H1N1	Q	K	S	T	Q	N	A	I	D	G	I	T	N	K	V	N	S	V	I	E	K
HK68 H3N2	L	K	S	T	Q	A	A	I	D	Q	I	N	G	K	L	N	R	V	I	E	K

D.



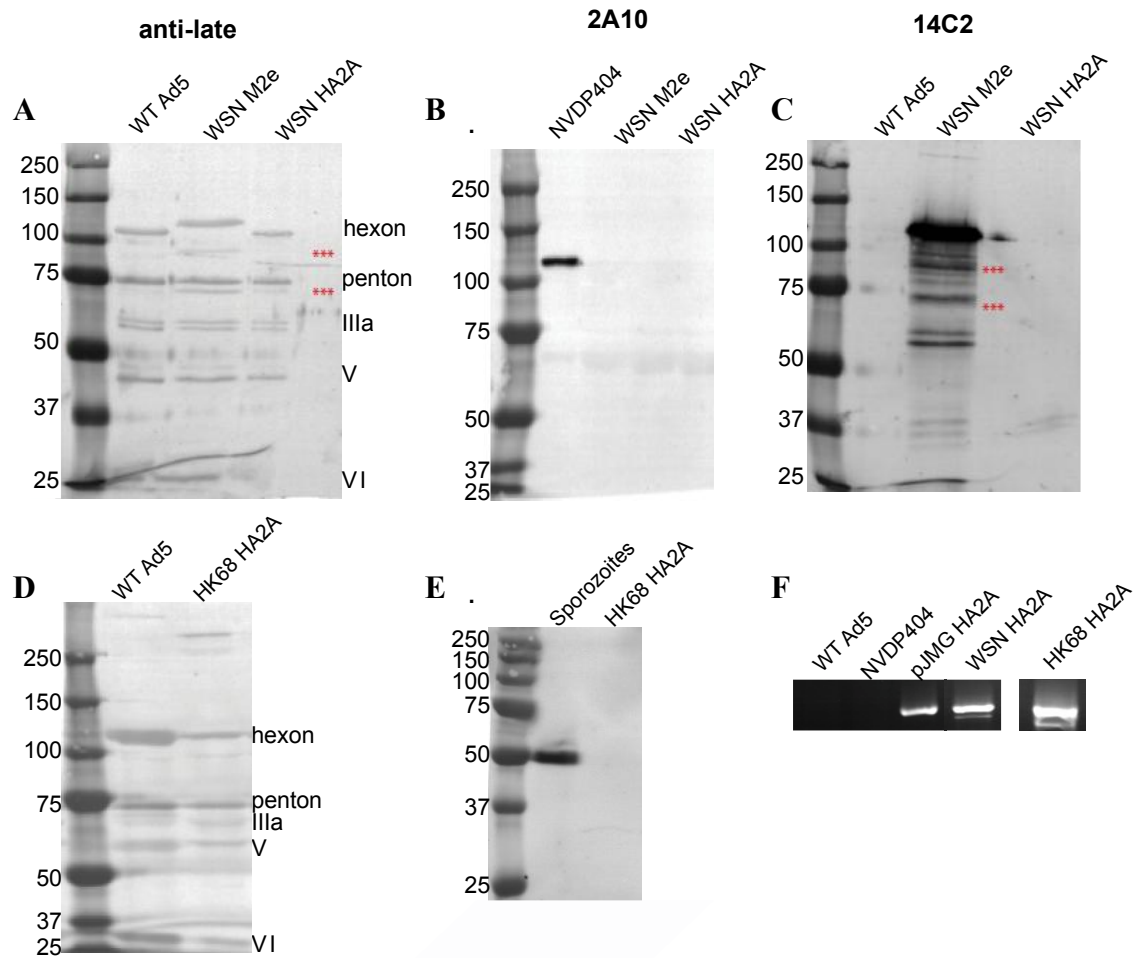
E.



**Figure 1: Conservation of the M2 ectodomain (M2e) and HA2 alpha (HA2A) helix amino acid sequence.**

A) Sequence alignment of the M2e, amino acids 2 to 24, for WSN H1N1 (human), 2009 pH1N1 and a representative swine H1N1 and avian H5N1. Amino acid differences between species are highlighted in yellow. B) Conservation of the M2e amino acid sequence, from residue 2 to 24, was compiled from all IAV strains. The percent M2e conservation was plotted against the consensus human M2e amino acid sequence on the x axis. C) Sequence alignment of the HA2A helix, amino acids 38 to 58 of HA2, for WSN H1N1 and X31 H3N2, representing the two HA clades. Amino acid differences between species are highlighted in yellow. Conservation of the H1 (D) and H3 (E) HA2A helix amino acid sequences, from residue 38 to 58, were compiled from all H1 or H3 virus strains. The percent HA2A conservation was plotted against the WSN H1 or X31 H3 HA2A amino acid sequence on the x axis.

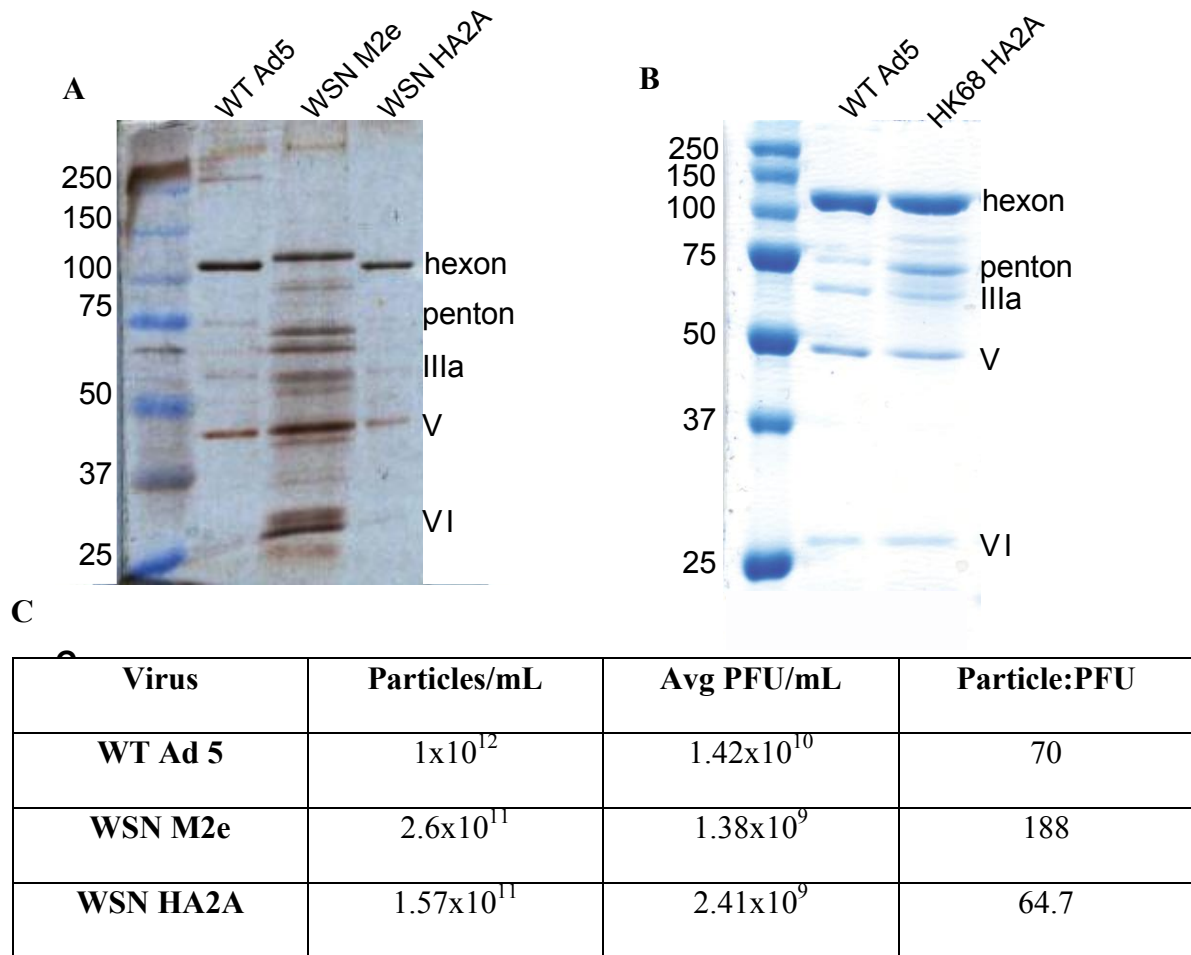




**Figure 2: Creation and confirmation of rAd constructs expressing M2e or HA2A.**

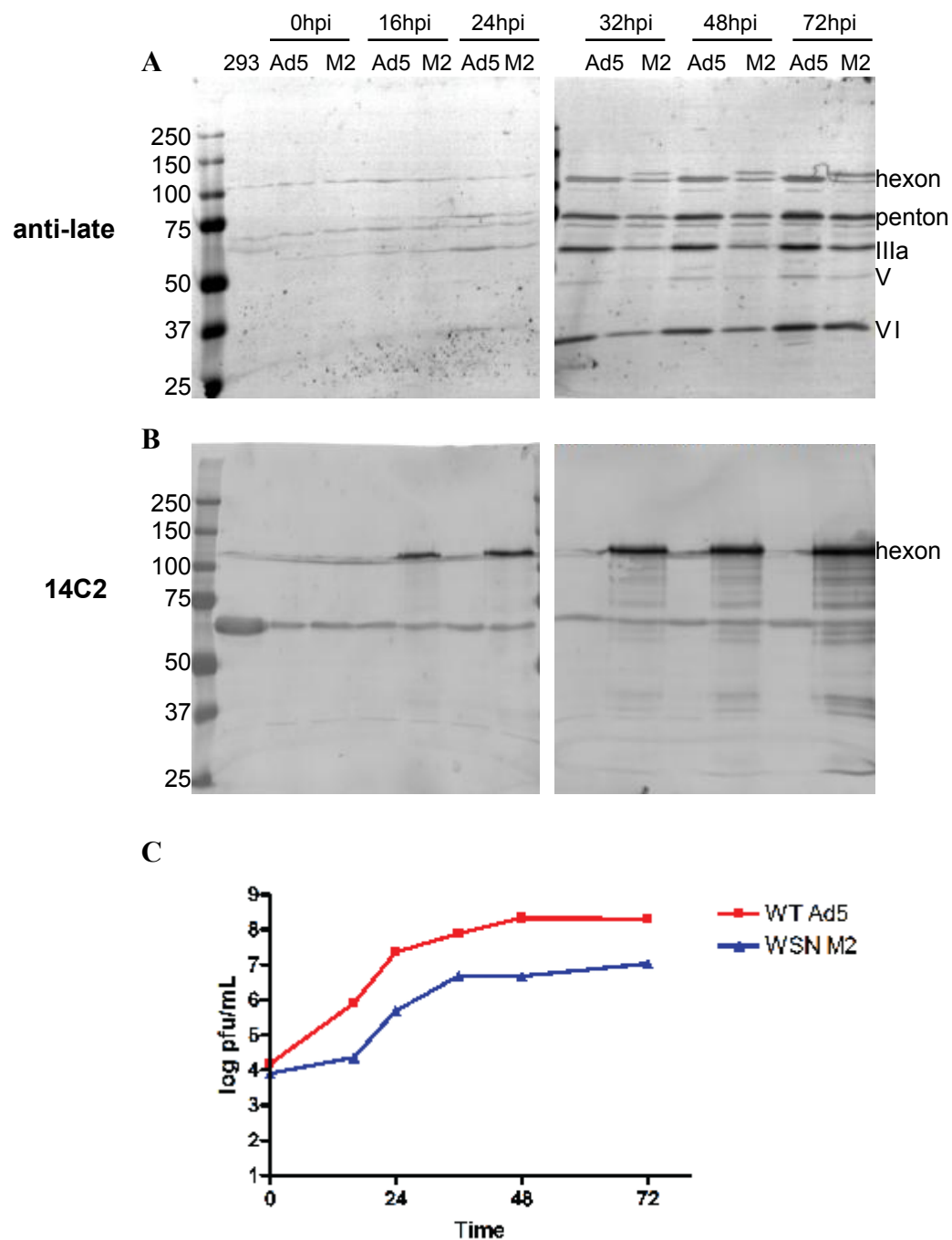
Infected cell lysates from plaque-purified recombinant viruses WSN M2e, WSN HA2A and HK68 HA2A, were analyzed for expression of the major capsid proteins with Ad5 anti-late serum (A and D), absence of the malaria CSP epitope with the 2A10 antiserum (B and E) and expression of the influenza conserved epitope M2e, using monoclonal 14C2 antibody (C) or by diagnostic PCR for HA2A (F). The positions of the major Ad capsid proteins are marked on the right of A and D. Three red stars in A and C indicate extra bands underneath hexon and penton that were detected by anti-late and 14C2 in the WSN M2e infected lysates. WSN HA2A and HK68 HA2A diagnostic PCRs (F) were performed with Ad5F and either WSN HA2A R or HK68 HA2A R primers, depending on the virus sample, but each resulted in the same size product. These PCRs

were performed at different times. The positive control was the corresponding pJMG HA2A DNA plasmid and the negative controls were WT Ad5 and NVDP404 viral DNA.



**Figure 3: Analysis of purified Ad particles expressing M2e or HA2A.**

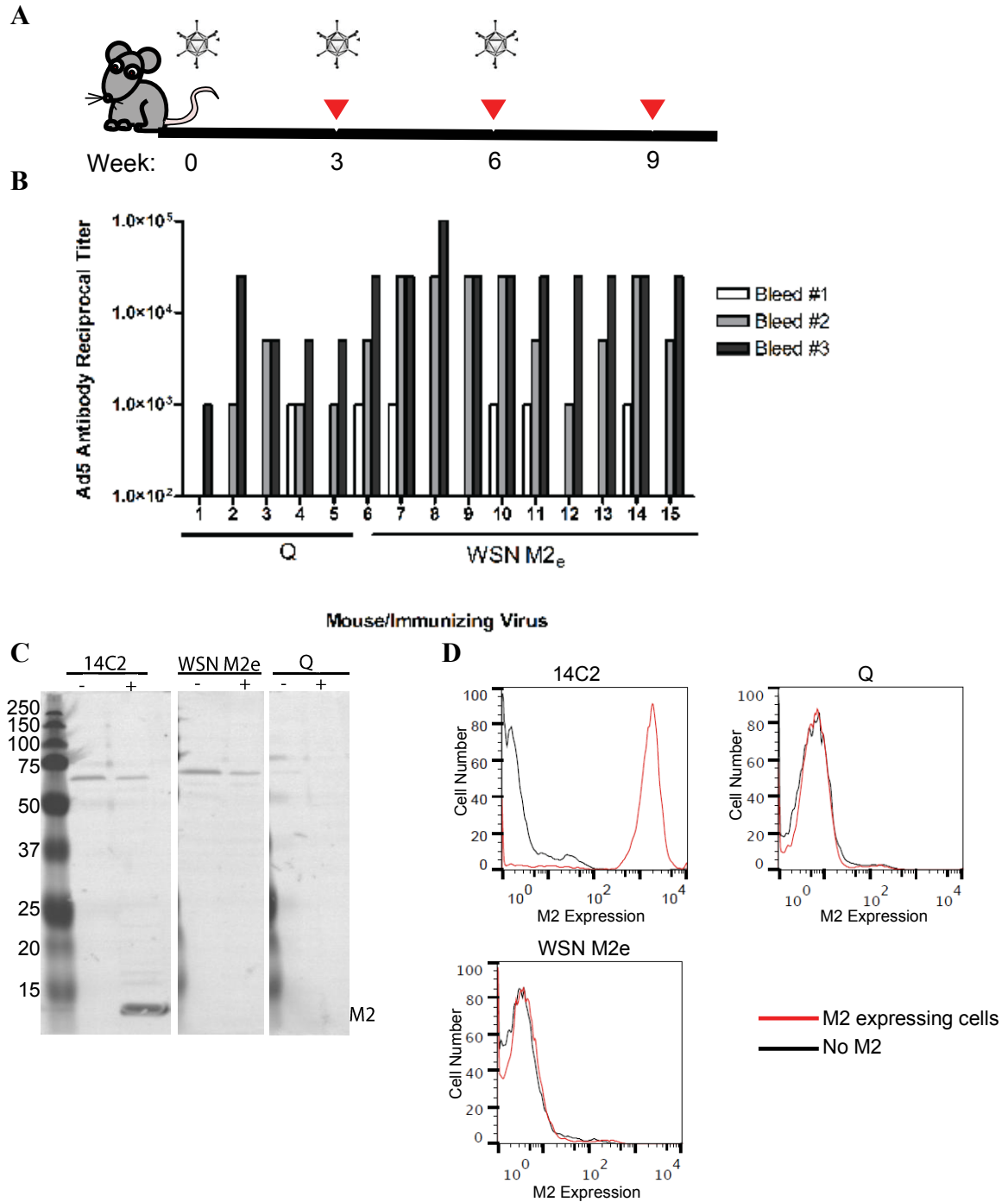
A) Silver stain of  $5.2 \times 10^9$  particles of WT Ad5, WSN M2e and WSN HA2A. B) Coomassie gel of  $1 \times 10^{10}$  particles of WT Ad5 and HK68 HA2A. The positions of the major Ad capsid proteins are indicated on the right. C) Table of the number of particles per mL, average plaque forming units (PFU) per mL and the particle to PFU ratio of WT Ad5, WSN M2e and WSN HA2A after cesium chloride virus purification. Virus purifications occurred on different days and are based on a different number of infected cells which would affect comparison of viruses based on the particles per mL and average PFU per mL. The particle to PFU can be used for comparison of the purified viruses.



**Figure 4: Protein production and growth kinetics of rAd WSN M2e.**

WSN M2e was further characterized by analyzing the protein production and growth kinetics through western blot (A and B) and titer by plaque assay (C) compared to WT Ad5. HEK 293 cells were infected with either WT Ad5 (Ad5) or WSN M2e (M2) viruses at a multiplicity of

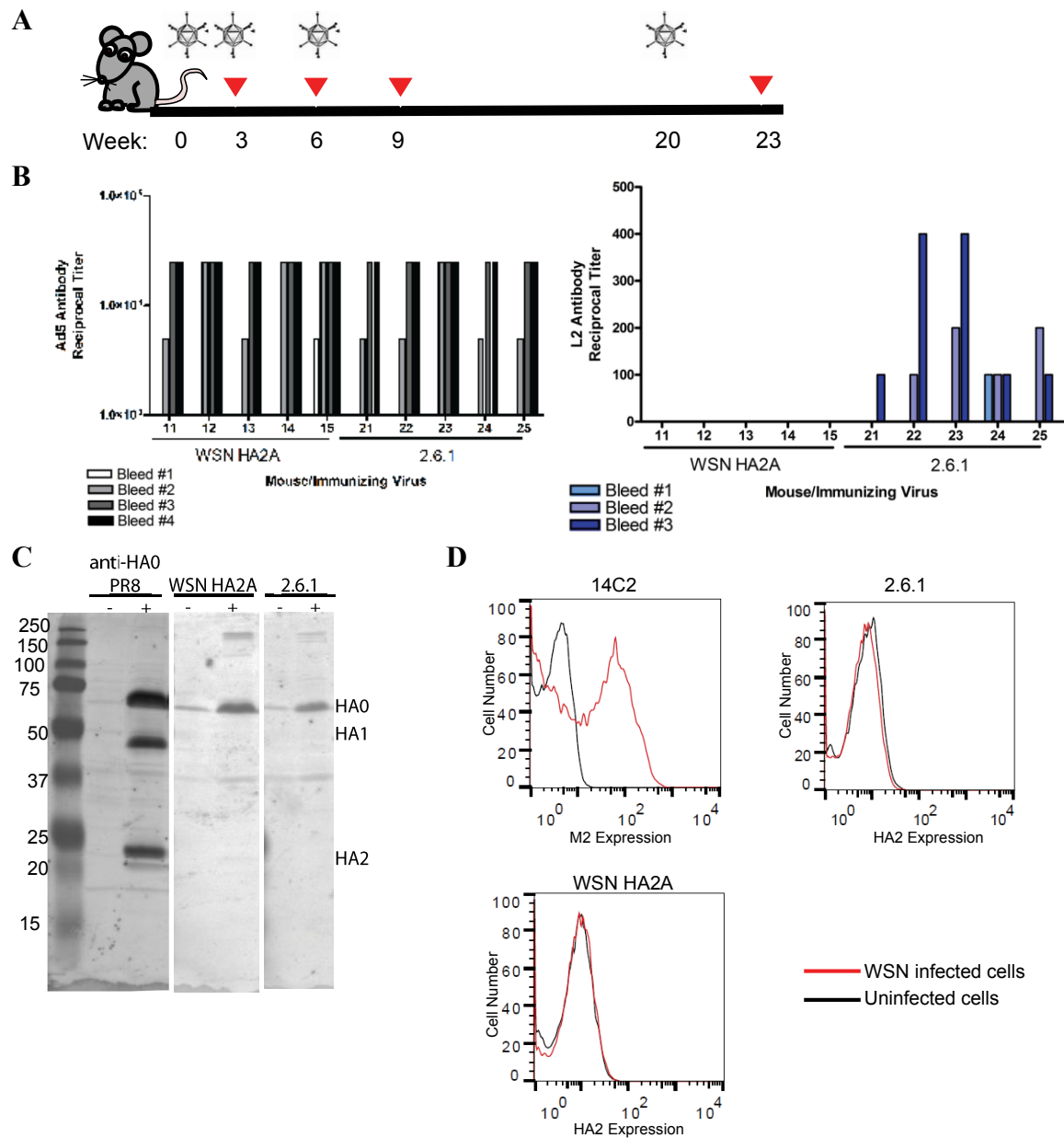
infection (MOI) of 5 and cell lysates were collected at the indicated time points. Samples were run on an SDS-polyacrylamide gel and proteins were probed with the Ad anti-late serum (A) to view the production of capsid proteins over time or 14C2 (B) to see the creation of 14C2-reactive hexon cleavage products over time. C) Samples were taken at 24, 36, 48 and 72 hours post infection (hpi) to determine the amount of infectious virus, presented as log pfu/mL, at each time point.



**Figure 5: rAd WSN M2e induces Ad5 antibodies but not M2e-specific antibodies.**

A) 15 female Balb/c mice were immunized by subcutaneous injection with  $1 \times 10^{10}$  particles of either recombinant virus WSN M2e or Q, a virus expressing the HPV16 L2 antigen in HVR1.

Mice were vaccinated and boosted twice at 3 week intervals and were bled prior to each boost or 3 weeks post boost. Ad particles indicate immunization with rAd and the red triangle denotes mouse tail bleeds. B) Mouse sera was analyzed for Ad5 antibodies by ELISA for both Q (mouse 1-5) and WSN M2e (mouse 6-15) for all bleeds. Sera was diluted in five-fold steps starting at 1:1000. Values were normalized against wells containing antigen but no sera. Sera was analyzed for reactivity against M2 by western blot (C) or flow cytometry (D) of M2 expressing cells or no M2. C) MDCK cells (-) and MDCK cells expressing M2 (+) were probed with the monoclonal M2 antibody 14C2 (left panel) or pooled sera from the third bleed of mice immunized with WSN M2e (middle panel) or Q (right panel) at 1:500. D) Representative flow diagram of M2 expression on M2 expressing versus non-expressing MDCK cells using 14C2 (1:500), and Q or WSN M2e immunized mouse sera from the third bleed (1:100). The plots come from the same experiment and each plot represents two cell types probed with the same antibody or sera. Each sera comes from one mouse but is representative of the immunized group.

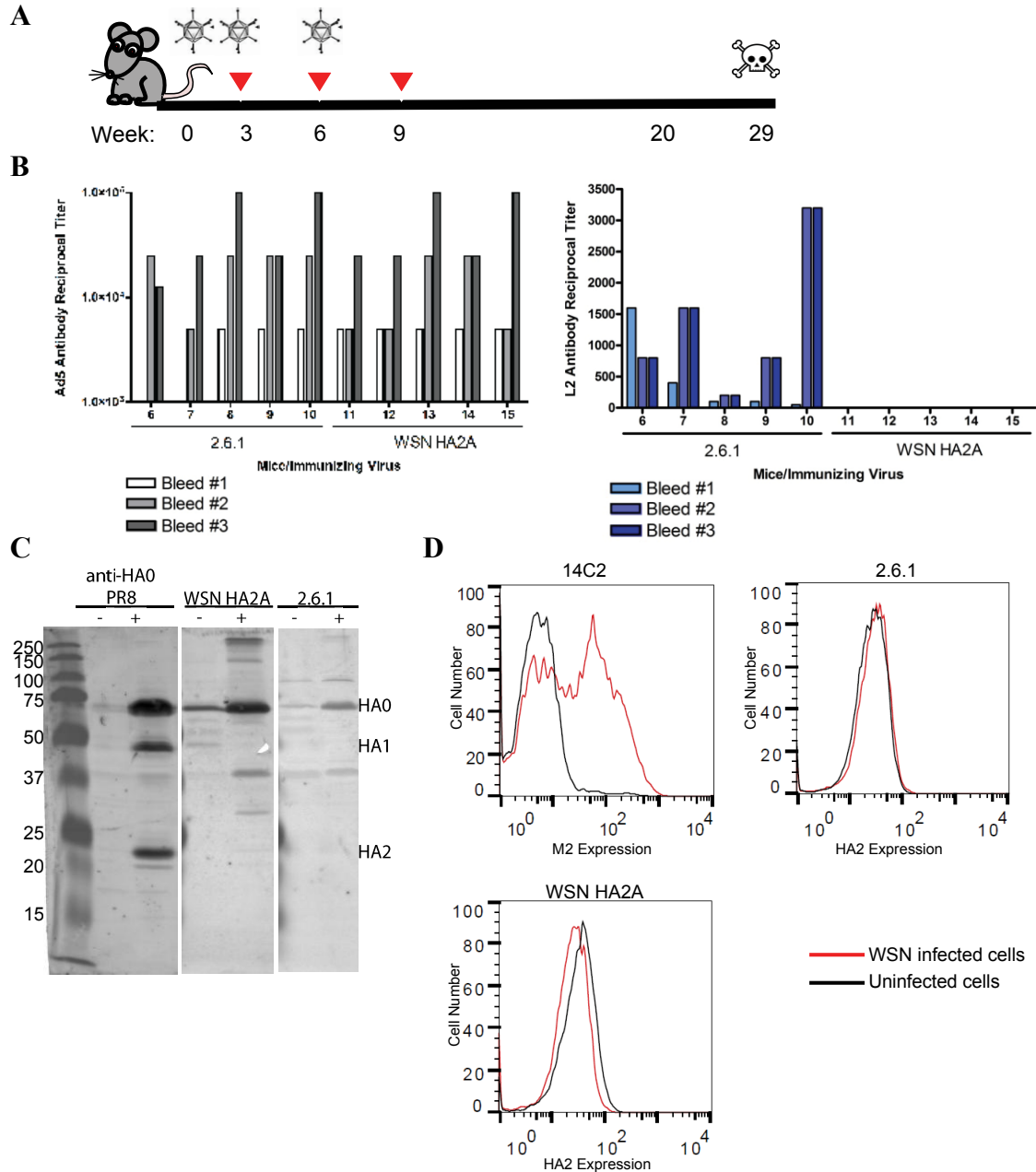


**Figure 6: rAd WSN HA2A induces Ad5 antibodies but not HA2-specific antibodies.**

A) 20 female Balb/c mice were immunized by subcutaneous injection with  $1 \times 10^{10}$  particles of either recombinant virus WSN HA2A or 2.6.1, a virus expressing HPV16 L2 antigen in HVR5. Mice were vaccinated and boosted twice at 3 week intervals with a third boost eleven weeks later. The first 5 mice of each group were bled prior to each boost or 3 weeks post boost. Ad particles indicate immunization with rAd and the red triangle denotes mouse tail bleeds. B) Mouse sera



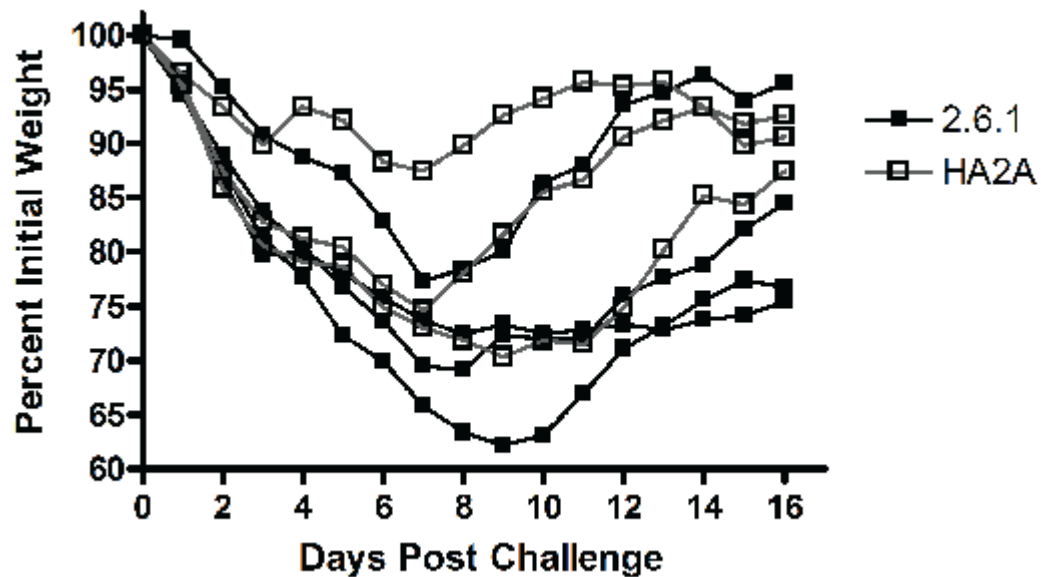
was analyzed for Ad5 (right) and L2 (left) antibodies by ELISA for both WSN HA2A (mouse 11-15) and 2.6.1 (mouse 21-25). Sera was diluted in five-fold steps starting at 1:1000 for Ad5 and in two fold dilutions starting at 1:50 for L2. Values were normalized against wells containing antigen but no sera. Bleed #4 was not assayed for L2 antibodies. Sera was analyzed for reactivity against HA2 by western blot (C) or flow cytometry (D) of uninfected or WSN infected cells. C) Uninfected (-) and WSN-infected (+) MDCK cells were probed with the polyclonal anti-HA0 PR8 antibody (left panel) or pooled sera from the third bleed of mice immunized with WSN HA2A (middle panel) or 2.6.1 (right panel) at 1:500. The positions of HA0, HA1 and HA2 are indicated on the right. D) Representative flow diagram of M2 or HA2 expression in uninfected versus WSN-infected MDCK cells using 14C2 (1:500) to confirm infection and 2.6.1 or WSN HA2A immunized mouse sera from the fourth bleed (1:100). The plots come from the same experiment and each plot represents two cell types probed with the same antibody or sera. Each serum comes from one mouse but is representative of the immunized group.



**Figure 7: MPL and alum adjuvants do not induce HA2-specific antibodies.**

A) 10 female Balb/c mice were immunized by subcutaneous injection with  $1 \times 10^{10}$  particles of either recombinant virus WSN HA2A or 2.6.1, supplemented with 50ug alum and 5ug MPL. Mice were vaccinated and boosted 2x at 3 week intervals and bled prior to each boost or 3 weeks post boost. Ad particles represent immunization with rAd, the red triangle denotes mouse tail

bleeds and the skull and crossbones represents influenza challenge at week 29. B) Mouse sera was analyzed for Ad5 (right) and L2 (left) antibodies by ELISA for both WSN HA2A (mouse 11-15) and 2.6.1 (mouse 6-10). Sera was diluted in five-fold steps starting at 1:1000 for Ad5 and in two fold dilutions starting at 1:50 for L2. Values were normalized against wells containing antigen but no sera. Sera was analyzed for reactivity against HA2 by western blot (C) or flow cytometry (D) of uninfected or WSN infected MDCK cells. C) Uninfected (-) and WSN-infected (+) MDCK cells were probed with the polyclonal anti-HA0 PR8 antibody (left panel) or pooled sera from the third bleed of mice immunized with WSN HA2A (middle panel) or 2.6.1 (right panel) at 1:500. The positions of HA0, HA1 and HA2 are indicated on the right. D) Representative flow diagram of M2 or HA2 expression in uninfected versus WSN-infected MDCK cells using 14C2 (1:500) to confirm infection and 2.6.1 or WSN HA2A immunized mouse sera from the third bleed (1:100). The plots come from the same experiment and each plot represents two cell types probed with the same antibody or sera. Each serum comes from one mouse but is representative of the immunized group.



**Figure 8: Adjuvant-supplemented vaccination with rAd WSN HA2A does not protect mice from a sub-lethal homologous influenza A challenge.**

10 female Balb/c mice immunized 3x with  $1 \times 10^{10}$  particles of either recombinant virus WSN HA2A or 2.6.1, supplemented with 50ug alum and 5ug MPL were challenged with 10 LD<sub>50</sub> of rWSN. Mice were weighed daily to monitor morbidity. The percent weight loss was plotted against the days post challenge for each individual mouse. Note: 2 rAd WSN HA2A and 1 2.6.1 mice died due to anesthesia prior to challenge.

<b>Epitope</b>	<b>Hypervariable Region Insertion</b>
WSN M <sub>2</sub> e	HVR1
Swine M <sub>2</sub> e	HVR5
Avian M <sub>2</sub> e	HVR1
2009 pH1N1 M <sub>2</sub> e	HVR5
WSN H1N1 HA2A	HVR5
X31 H3N2 HA2A	HVR5

**Table 1: Recombinant capsid-display vaccines expressing M2e or HA2A sequences in either the hypervariable region (HVR) 1 or HVR5 of hexon.**

Ad5-influenza recombinant constructs with the epitope and insertion location within hexon.

Influenza Epitope Insert on Primers		
Name	Function	Sequence
WSN HVR1 F	WSN M2e insertion into HVR1	gcctatcagaacgaatgggggtgcagatgcaacgattcaagtgtatcCTGC TACTGCTCTTGAAATAAACC
WSN HVR1 R	WSN M2e insertion into HVR1	gcacccccattcgtt ctgataggcgtt cgacctcggtcagaaggctAGCTT CATCCCATTCGCAAGG
Avian HVR1 F	Avian M2e insertion into HVR1	ctaccagaaacgaatgggagtgagatgcagcgattcaagtgtatcCTGCTA CTGCTCTTGAAATAAACC
Avian HVR1 R	Avian M2e insertion into HVR1	gcactccattcgtt ctgtaggcgtt cgacctcgtt agaagactAGCTT CATCCCATTCGCAAGG
Swine HVR5 F	Swine M2e insertion into HVR5	ctaccagaaacgaatgggagtgagatgcagatcagcgattcaagtgtatcCTGCA CCGAGGCAATGGTG
Swine HVR5 R	Swine M2e insertion into HVR5	gcactccattcgttctgtaggcgtt cgacctcgtt agaagactCTCAG TAGTTGAGAAAAATTGCATTCCAC
2009 H1N1 HVR5 F	2009 H1N1 M2e insertion in HVR5	ctaccagaaacgaatgggagtgagatgcagcgattcaagtgtatcCTGCA CCGAGGCAATGGTG
2009 H1N1 HVR5 R	2009 H1N1 M2e insertion in HVR5	gcactccattcgtt ctgtaggcgtt cgacctcgtt agaagactCTCAG TAGTTGAGAAAAATTGCATTCCAC
WSN HA2A HVR5 F	WSN HA2A insertion in HVR5	gccatcgacaaatcaatgggaaatgaacagggtatcgagaagGCGAC CGCAGGCAATGGTG
WSN HA2A HVR5 R	WSN HA2A insertion in HVR5	ctgtt gtaatccgtt aatggtt ctgtgtttt gCTCAGTAGTTG AGAAAAATTGCATTCCAC
HK68 HA2A HVR5 F	HK68 HA2A insertion in HVR5	cgacaaatcaatgggaaatgaacagggtatcgagaagGCGACCGCA GGCAATGGTG
HK68 HA2A HVR5 R	HK68 HA2A insertion in HVR5	ccctgtcaatt ccatgat tt ggtcgatggcgtt gagtt ttt aagCTC AGTAGTGAGAAAAATTGCATTCCAC
Sequencing and Diagnostic PCR Primers		
Name	Function	Sequence
JMG1 (Ad5F)	Universal hexon forward binding at beginning of hexon	CGGCGTGTGGACAGGGGCC
JMG4 (Ad5R)	Universal hexon reverse binding at end of hexon	GCTGGCTCCGTCAACCC
HVR1R	Universal hexon reverse binding in HVR1	CGTCTTCGTTGTCATCG
HVR4F	Universal hexon forward binding in HVR4	GGAGGGCAAGGCATTC
HVR5R	Universal hexon forward binding in HVR5	GTTATCACCATTGCCTGC
HVR6R	Universal hexon reverse binding in HVR6	GTTGGCCATTAGTTCTCG
WSN HA2A R	WSN HA2A primer for diagnostic PCR	ctcgataacagagttcacc
HK68 HA2A R	HK68 HA2A primer for diagnostic PCR	cttctgat accctgt c

**Table 2: Primers used for sequencing and insertion of M2e and HA2A epitopes**

List of name, function and sequences of primers used for either insertion of influenza epitopes into HVR1 or HVR5 of Ad5 hexon or for sequencing and diagnostic PCR. All sequences present in capital letters denote Ad5 sequence and lowercase letters denote influenza sequences.

<b>Virus</b>	<b>Epitope</b>	<b>Hypervariable Region Insertion</b>
<b>WSN M<sub>2</sub>e</b>	WSN M <sub>2</sub> e	HVR1
<b>WSN HA2A</b>	WSN HA2A	HVR5
<b>Q</b>	HPV16 L2	HVR1
<b>2.6.1</b>	HPV16 L2	HVR5

**Table 3: rAds used for mouse immunizations.**

List of rAds, the epitope expressed and insertion location with hexon that were used for mouse immunizations and challenges.

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## **Chapter Five:**

### **Current status of malaria vaccines and adeno-associated virus vectors**

Despite decades of research, malaria continues to be a highly significant global health problem and the most important tropical parasitic disease, accounting for the deaths of more children under five than any other disease [1]. Among infectious diseases, malaria ranks fourth as a cause of death and in Africa in 2010, the WHO estimates that there were nearly 3 million clinical cases and roughly 700,000 deaths [2]. However, recent analysis suggest that this figure greatly underestimates the true burden of disease, with the global death toll actually exceeding 1.2 million [3]. Malaria is a disease of the impoverished, having the greatest impact on the world's poorest countries. More than 80% of malaria cases and 90% of malaria-related deaths occur in sub-Saharan Africa [2]. The malaria burden is further increased by enhanced susceptibility to other infections and life-long health effects due to prenatal malaria exposure [4]. It has been reported that malaria incidence has been reduced by approximately 17% since 2000, with mortality rates by 26%, through efforts of malaria control involving insecticides to control the mosquito vector, indoor residual spraying programs, antimalarial drugs and treated bednets to reduce exposure to infected mosquitoes [2]. However, these efforts will be difficult to sustain in the face of growing drug resistance among parasites, insecticide resistance in the vector population and economic difficulties due to reduced malaria control funding [2]. Currently, a licensed vaccine against malaria does not exist and many promising experimental malaria vaccines have failed to protect or only partially protect residents of malaria-endemic areas. Research shows this may be in part due to B and T cell exhaustion resulting from chronic exposure to malaria [5]. The most advanced candidate, RTS,S, which aims to prevent infection, confers only partial protection. Eradication will not be feasible with our current existing tools and knowledge. In order to maintain sustainable reductions in malaria morbidity and mortality and with the final aim of eradication, the development and implementation of an effective vaccine is paramount.

There are many lines of evidence that indicate that a vaccine could be made to protect against malaria. Individuals born in endemic areas who survive the first year of exposure continue to develop parasitemia but are resistant to the severe, life-threatening malaria that plague children and eventually, become resistant to clinical disease [6]. However, to maintain this level of protection, frequent re-exposure is required and sterile immunity rarely if ever develops naturally. Clinical protection from malaria is possible as gamma-globulin from semi-immune individuals can mitigate malaria disease in naïve humans [7]. Researchers have even achieved sterile immunity by inoculating humans with irradiated sporozoites by mosquito bite [8, 9]. This has been shown to reproducibly prevent the emergence of blood-stage parasites that are associated with clinical disease. However, this approach presents many practical challenges, requiring research into different vaccination methods.

The development of an effective vaccine has been hindered by many challenges, primarily through the complexity of the immuno-evasive parasite and its multi-stage life cycle. Malaria is a protozoan parasite of the genus *Plasmodium* of which four different species can cause malaria in humans: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* [2]. Recently a fifth species, *P. knowlesi*, a parasite infecting wild macaques, has been detected in humans [10]. While disease caused by infection with *P. malariae* and *P. ovale* is less severe and rarely fatal, infection with *P. falciparum* and *P. vivax* can lead to serious disease outcomes such as anemia, encephalopathy, coma, respiratory distress and organ failure, usually resulting in death. Of these parasites though, *P. falciparum* is responsible for greater than 90% of disease [2].

Infection by all malaria species starts with the bite of an infectious female *Anopheles* mosquito, that upon blood feeding, injects sporozoites, the motile stage of the parasite, into the host (Figure 1). A relatively small number of sporozoites are injected into the skin, approximately 10 to 200, and they rapidly enter the bloodstream in order to migrate to the liver, a process which can take minutes to hours. Within the liver, sporozoites invade hepatocytes where

they develop into their exoerythrocytic form over one to two weeks; this stage marks the pre-erythrocytic stage and ends when hepatocytes rupture, releasing thousands of merozoites into the blood stream. Merozoites mark the erythrocytic stage of the parasite life cycle and upon invasion of red blood cells (RBCs), they undergo asexual development from rings to trophozoites, then dividing to form schizonts. These schizonts rupture RBCs, releasing merozoites which then infect new RBCs. The repeated cycling and infection of RBCs is responsible for the cyclical fever associated with malaria infection and leads to the majority of pathology. Less than 5% of parasites in the blood undergo sexual differentiation to form gametocytes [11]. These are ingested by a mosquito during a blood meal where they undergo sexual reproduction forming zygotes which develop into oocysts. Oocysts develop in the hemocoel of basal lamina of the mosquito midgut and upon rupturing, release sporozoites into the hemocoel. This allows the sporozoites to traffic to the salivary glands of mosquitoes, thus rendering the mosquito infectious and completing the parasite life cycle.

### **Targets of current malaria vaccines**

Current malaria vaccines are directed against the 3 different stages of the *Plasmodium* life cycle: the pre-erythrocytic stage of sporozoites and liver invasion, the erythrocytic phase involving the invasion and asexual reproduction within RBCs and finally, the transmission stage involving the uptake of gametocytes and further development of the parasite within the mosquito (Figure 1) [2]. In general, pre-erythrocytic vaccines are designed to induce an immune response against sporozoites and/or the liver stage through the induction of antibodies that will inhibit sporozoite motility and prevent invasion of hepatocytes and/or the induction of CD8<sup>+</sup> T cells that will recognize and kill infected hepatocytes. Effective vaccines targeting this stage would completely prevent the asexual and sexual blood stages from developing thus preventing the manifestation of disease and transmission of the parasite. The predominant targets of these vaccines that are under current development include the circumsporozoite protein (CSP) that is

the major surface antigen of sporozoites, thrombospondin-related adhesion protein (TRAP) involved in motility and sporozoite invasion [12, 13], and liver-stage antigen (LSA) involved in liver stage differentiation [14-16]. Importantly, this stage represents a bottleneck in the malaria life cycle, where only a small number of sporozoites are injected upon mosquito bite leading to few infected hepatocytes, making this type of vaccine extremely advantageous as only a limited number of parasites need to be controlled in order to be successful. However, every single parasite needs to be neutralized in order to achieve sterile immunity.

Unlike pre-erythrocytic vaccines, blood stage (erythrocytic) vaccines do not prevent infection but reduce severity of the disease by decreasing the number of parasites in the blood. Studies have suggested that individuals who survive regular exposure to malaria develop this immunity over time although this immunity is not sterile [17, 18]. These vaccines aim to accelerate the development of this immunity by inducing antibodies that prevent merozoite invasion of RBCs or target antigens expressed on the surface of RBCs. While there are hundreds of potential targets expressed by the blood-stage parasites, several targets have been utilized in the development of erythrocytic vaccines including merozoite surface protein (MSP-1), MSP-2, MSP-3, apical membrane antigen 1 (AMA-1), serine repeat antigen 5 (SERA5), and *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) [19]. However, while several of these antigens appear to be protective in a non-human primate (NHP) model [20-23], none have achieved protective efficacy against clinical malaria as there is significant diversity of these targets among *P. falciparum* isolates which limits their utility in the field.

Lastly, transmission blocking vaccines target the sexual gametocytes within the human host and antigens expressed during the developmental stages within the mosquito vector. These vaccines would not confer protection directly to the vaccinee but prevent infection of the mosquito host in order to lower the parasites transmission efficiency, thus eliciting herd immunity within the general population. Antibodies induced against antigens such as the *Plasmodium*

ookinete surface protein Pfs25 [24-26] would be ingested by a feeding mosquito and would prevent the parasite from developing into infectious sporozoites within the mosquito. While attempts at developing transmission blocking vaccines have demonstrated protection in an animal model, they have demonstrated poor immunogenicity in humans thus far [27].

### **Rationale for pre-erythrocytic vaccines**

Pre-erythrocytic vaccines have the potential to prevent clinical disease and transmission, thus making it one of the most advantageous vaccine targets for malaria control. Protective immunity against sporozoites has been induced by inoculation with radiation-attenuated sporozoites in both animal models and human volunteers [9, 28-30]. While irradiated sporozoites are the most effective and only approach to induce sterile immunity to date, there are significant logistical limitations that have prevented licensure of this vaccine. Sterile protection in humans by irradiated sporozoites can either be induced by the bite of more than 1000 irradiated infected mosquitoes [9] or through five intravenous (i.v.) injections of irradiated sporozoites [29]. Both of these methods are tedious and labor intensive, requiring sterility and cold-chain storage to ensure sporozoite viability; i.v. injections also require administration by highly-trained personnel. The clinical feasibility of meeting all of these requirements in areas where malaria is a significant public health problem such as Africa or India, is very slim.

While logistical problems are associated with irradiated sporozoite vaccines, the knowledge that they are capable of providing sterile immunity has helped advance the field of malaria pre-erythrocytic vaccines. By focusing on the immune response that is elicited by irradiated sporozoites, it has been possible to better understand the protective aspects of this immune response. Research has demonstrated that the predominant immune response in sporozoite vaccinated volunteers was antibody directed against CSP [31]. CSP is a protein that covers the surface of sporozoites and is involved in many important functions during the

sporozoite stage of the *Plasmodium* life cycle. In the mosquito vector, it aids in sporozoite formation and egress from oocysts along with sporozoite invasion of the salivary glands [32, 33] while in the human host, CSP helps with hepatocyte invasion and parasite development [34]. It was actually the first *Plasmodium* gene to be cloned and provided evidence for antibody-based protection in mice [35]. Further studies demonstrated that the majority of anti-CSP antibodies recognized a common epitope (NANP)<sub>3</sub> [35-38]. This central NANP repeat B cell epitope is species-specific but is highly conserved across *P. falciparum* isolates from different geographical regions, although these isolates can differ in CSP size due to the variable number of repeats [37]. Antibodies raised against synthetic NANP peptides have been shown to prevent sporozoites from invading hepatocytes *in vitro* [38] and passive transfer of monoclonal antibodies against this epitope can completely protect from disease in both a mouse and NHP model [39]. Thus, this immunodominant B-cell epitope is commonly used in current malaria vaccines and the ability to elicit the formation of antibodies that recognize these repeats has been shown to be a crucial component of effective pre-erythrocytic vaccines [40, 41]. However because of the short period of time that sporozoites are exposed to antibodies in the skin and blood stream before invading hepatocytes, a high sustained level of CSP-specific antibodies are required for a solely humoral-based protective immune response.

CSP not only contains a central B-cell epitope but also contains both conserved and variable T cell epitopes [42]. These regions serve to induce CD8<sup>+</sup> T cells that are capable of killing sporozoite-infected hepatocytes. While a majority of research has focused on the importance of antibodies in protection from malaria, it is thought that a cell-mediated immune response is also important as CD8<sup>+</sup> T cell-ablated mice did not respond as readily to immunization with sporozoites [43, 44]. T cell responses are HLA-type dependent but examination of irradiated sporozoite vaccinated volunteers uncovered a universal T-cell epitope, known as T\*, that is independent of the HLA class II composition of the host [45]. This T-cell



epitope has been shown to induce a protective immune response in animals and immunized volunteers [46, 47], becoming another major component of many sporozoite-based vaccines.

### **Current pre-erythrocytic vaccine candidates in clinical trials**

While many attempts have been made to induce an immune response against the pre-erythrocytic phase for a malaria vaccine, only one has reached a phase III clinical trial. This vaccine, RTS,S, began as a collaborative effort between GlaxoSmithKline (GSK) and the Walter Reed Army Institute of Research (WRAIR) in the 1980's. They initially attempted to increase the immunogenicity of CSP as a T cell epitope by fusing the C terminus containing 19 CSP NANP repeats and a T cell epitope to the hepatitis B surface protein (HBs) and formulated it with excess HBs, forming virus-like particles (VLP) [48]. The reasoning was the belief that particulate antigens were processed and presented in a more immunogenic manner than soluble proteins. This vaccine was not effective until an oil-in-water adjuvant, ASO1, was added, which was later optimized to the liposomal MPL and QS21-bearing adjuvant ASO2 [49, 50]. RTS,S was shown to be ~50% efficacious against a homologous challenge in malaria-naïve individuals [51] and further extensive field trials confirmed that it prevented 26% of clinical malaria and 58% of severe malaria immediately after vaccination in children 5-17 months of age [46, 52, 53]. Subsequent data demonstrated that while RTS,S was also effective in preventing clinical malaria in adults, the vaccine only provides protection after multiple doses and for a limited period of time, with protection waning after several months, dropping to ~16% efficacy within 4 years [46, 52-56]. Protective efficacy of the vaccine is highly region specific and protection may actually be only 30% within 6-12 week old babies [57]. The disappointing protective efficacy may rise from its relatively poor induction of a cell mediated response and waning antibody titers. However, despite these results in the Phase III trial, RTS,S may still be recommended for licensure and implementation because of its anticipated ability to reduce severe malaria in children [58]. Surprisingly, although this vaccine only targets the pre-erythrocytic stages of the parasite life

cycle, there was a reduction in the incidence of uncomplicated and severe malaria amongst vaccinated children [59]. It is possible that the vaccine is significantly reducing the number of liver-stage shizonts reaching maturity, thereby allowing the host immune system more time to mount an effective immune response to limit blood-stage parasite replication. From a public health standpoint, even a small amount of protection for a short period of time could be useful.

While RTS,S induced anti-CSP IgG titers and a modest CD4<sup>+</sup> T cell response, the induction of a CD8<sup>+</sup> T cell response was low or completely absent [60]. This is in contrast with what is observed with viral vectored vaccines, specifically adenovirus (Ad) vectors. A key attraction of using this vector for vaccination against malaria is the potential to prime all aspects of the immune response to induce a far greater cell-mediated response without the use of adjuvants. Thus, many Ad-vectored malaria vaccines have entered clinical trials [61]. Initially, most research was conducted using the Ad serotype 5 (Ad5) vector that is the best characterized and lends itself well for use in preliminary studies. However, there are many disadvantages to this serotype as it is the most common human serotype with greater than 50% of the population in Africa having neutralizing antibodies to Ad5 [62, 63]. The potential interference of pre-existing immunity on the efficacy of an Ad5-vectored malaria vaccine is unknown however it is thought to be disadvantageous as the antibodies would prevent initial cell infection by the virus and would reduce the CD8<sup>+</sup> T cell response, thus reducing the vaccine efficacy [64-67]. Concerns about using this serotype were exacerbated by the HIV STEP trial where a recombinant Ad5-vectored HIV vaccine demonstrated a high rate of HIV infection in the vaccinated group with an increased risk of HIV infection post-vaccination in uncircumcised males or those with high pre-existing neutralizing Ad5 antibodies [68, 69]. Although subsequent investigation has failed to establish a causal link between pre-existing Ad5 immunity and HIV infection [70], the HIV-STEP trial still represents a roadblock in using Ad5 for prophylactic vaccines. Fortunately, other Ad serotypes exist that have a lower seroprevalence in the population such as Ad35 [71] and AdCh63, a

chimpanzee serotype. A non-replicating Ad35-vectored CS vaccine induced strong IFN- $\gamma$  and CD8+ T cells responses in mice [72, 73]. This has been moved into Phase 1a and 1b clinical trials to be used in combination with RTS,S as a prime-boost approach with the hopes of improving RTS,S efficacy through the induction of CD8+ T cells [74].

Some vaccines use multiple pre-erythrocytic epitopes (ME) to broaden the immune response and prevent development of blood-stage infection. One example is the AdCh63/MVA ME-TRAP vaccine that has thus far been highly immunogenic. This vaccine is comprised of a pre-erythrocytic fusion antigen of 17 B cell, CD4+ and CD8+ epitopes from six different *P. falciparum* antigens, including CSP, fused to thrombospondin-related adhesion protein (TRAP), a protein that is expressed on sporozoites and in infected hepatocytes [75]. The rationale for this vaccine was the concept of IFN $\gamma$ -mediated killing of infected hepatocytes. During pre-clinical development, the AdCh63 ME-TRAP prime followed by the MVA (modified vaccinia virus Ankara) ME-TRAP boost elicited strong, long-lasting, polyfunctional CD8+ T cells [75, 76]. In this situation, a heterologous boost, being MVA, was utilized to prevent acquired immunity from the AdCh63 prime to interfere with the boost. Heterologous prime-boost approaches have been utilized frequently and with great success. They are particularly promising when a desired outcome is the induction of a strong CD8+ T cell response. Currently, a phase I adult Kenyan and phase Ib field trial in Gambia have occurred and the vaccine was found to induce high levels of T cells, with a median of greater than 1300 spot forming units (SFU) per million peripheral blood mononuclear cells (PBMC) as assessed by IFN $\gamma$  ELISPOT [77]. This vaccine has now moved onto a phase I study in the UK and a phase 2 sporozoite challenge study [74].

Another ME approach has recently entered clinical trials that contains antigens from four pre-erythrocytic proteins: CSP, TRAP, LSA-1 and exported protein-1 (EXP-1). This is a DNA vaccine administered through electroporation and is currently being assessed in Phase Ia clinical trials in naïve US volunteers [74]. Although this trial started in 2010, little information or details

have become available. While electroporation has augmented immune responses in animal models [78] and in an HIV trial [79], typically the immunogenicity of DNA vaccines in animals has not been reproduced in humans.

Most of the pre-erythrocytic vaccines currently in clinical trials attempt to provide protection by inducing an immune response against at least CSP and/or TRAP. However, another antigen has emerged as a potential target for vaccines, the cell-traversal protein for ookinetes and sporozoites (CelTOS) [80, 81]. This protein plays an important functional role in the cell traversal in both the mosquito vector and human host. It is very highly conserved among *Plasmodium* species thus it represents an important target for the immune response to interfere with the malaria life cycle. Currently, there is one phase Ia clinical trial using recombinant CelTOS expressed from *Escherichia coli* [74]. Preclinical characterization studies in mice demonstrated that vaccination with PfCelTOS resulted in potent humoral and cellular immune responses and, importantly, induced sterile protection in mice from a heterologous challenge with a *P. berghei* strain [82]. Induced antibodies targeting this protein likely function by inhibiting motility of sporozoites and preventing hepatocyte invasion. This is the first pre-erythrocytic candidate malaria vaccine that is cross-protective between malarial species and represents a very important step forward in the quest for an effective malaria vaccine.

There have been many successes on the road to developing a malaria vaccine with RTS,S being the first one to ever reach Phase III clinical trials. The Malaria Vaccine Technology Roadmap set goals in 2006 for the development of malaria vaccines with the eventual goal of eradication. First, to have a licensed vaccine with greater than 50% efficacy lasting for a year against severe disease and mortality by 2015 and second, to develop a vaccine by 2025 that induces protective efficacy against clinical disease of more than 80% lasting for over four years [83]. While a licensed RTS,S vaccine may meet the first goal, it fails to meet the requirements of the second goal, necessitating continued research on malaria vaccines. Conventional vaccination

approaches, to date, have not worked to make an 80% effective malaria vaccine. However, current knowledge about protection from malaria may still inform efforts to create a completely different approach: induction or “unnatural” immunity. Instead of relying on the immune system to develop a protective response against malaria, a protective component of the immune system, such as antibodies, can be given directly as purified monoclonal antibody (mAb) or via a viral vector, thereby harnessing engineered immunity and thus bypassing the uncertain pathway of immune induction by conventional antigens.

### **Adeno-associated virus**

The potential application of recombinant adeno-associated virus (rAAV) as gene transfer vectors was realized over three decades ago. Today, numerous preclinical studies have demonstrated the efficacy of utilizing rAAV vectors for gene therapy and vaccine purposes. AAV was first discovered as a contaminant of Ad stocks in the 1960's [84] and knowledge of basic AAV biology and its diverse tissue tropism helped to drive the development of rAAV vectors, with these vectors being used most frequently in gene therapy. The development of rAAV in gene therapy is in large part due to the lack of pathogenicity of the wild-type (WT) virus, ability to establish long-term transgene expression, ability to transduce both dividing and nondividing diverse cell types and low immunogenicity. Within the first 10 years of AAV use in humans, rAAV1 and -2 vectors were in clinical trials to treat cystic fibrosis, hemophilia B, Canavan's disease and  $\alpha$ -antitrypsin (AAT) deficiency [85]. The first breakthrough in clinical efficacy in humans was made when data demonstrated that patients treated with rAAV2-RPE65 to combat Leber's congenital amaurosis, an autosomal recessive disease resulting in functional blindness due to inability to recycle retinoids in the visual cycle, resulted in long-term improvements in sensitivity to light and improvements in vision [86-89]. Hemophilia B trials continued to progress with the use of an rAAV-factor IX vector that was delivered systemically for liver transduction [90]. Patients demonstrated a significant reduction in need for recombinant

protein replacement. Children with Parkinson's disease and congenital aromatic amino acid decarboxylase (AADC) deficiency showed improvement in gross motor development, including the ability to walk by 16 months in a single patient, upon treatment with an rAAV2-AADC vector [91, 92].

It was quickly realized that AAV serotypes had varying transduction efficiencies for certain tissues. AAV1 capsids were more effective at transducing skeletal muscle than AAV2 [93] whereas AAV5 and -6 were more efficient at transducing murine airway epithelia [94]. This knowledge and the ability to pseudotype vector genomes with AAV2-ITRs into capsids of other serotypes led to the engineering of more effective vectors [95], leading to the development of rAAV1-lipoprotein lipase (LPL) that was used to treat lipoprotein lipase deficiency. Intramuscular injection of this vector resulted in lowering of serum cholesterol levels and pancreatitis in LPL patients [96]. rAAV1-LPL was licensed in Europe in November 2012 [97, 98], becoming the first licensed AAV-vectored gene therapy and thus demonstrating the power and potential of this virus for gene therapy, vaccines and other therapeutic applications.

### **AAV structure and replication**

AAV is a helper-dependent DNA virus of the *Dependovirus* genus of the parvovirus family that depends upon helper functions supplied by coinfecting helper viruses such as Ad or herpes simplex virus (HSV) for productive infection. Like all members of the parvovirus family, AAV is a small (~25nm) nonenveloped icosahedral capsid comprised of three capsid proteins: VP1, VP2 and VP3. These proteins share a C-terminal region that forms the T=1, icosahedrally symmetric portion of the capsid. Each VP subunit consists of a conserved core of an eight stranded,  $\beta$ -barrel motif and an  $\alpha$ -helix [99]. The outer surface of the capsid is formed by large loops that connect the  $\beta$ -barrel strands. These loops are comprised of nine conformationally variable regions, designated VRI-VRIX [100]. The variable interstrand loops of this motif

determine receptor usage, transduction efficiency and serology. VPI also contains an N-terminal conserved phospholipase A2 sequence that is crucial for infectivity as it is implicated in viral escape from endosomes [101].

Within the nonenveloped capsid, the AAV genome is single-stranded DNA of approximately 4.7kb, flanked by inverted terminal repeats (ITRs) that form T-shaped, base-paired hairpin structures with *cis*-elements required for replication and packaging. The genome contains three viral promoters (p5, p19 and p40) and codes for two genes, *rep* and *cap*, which encode four nonstructural Rep proteins (Rep78, -68, -52, and -40) required for replication and the three structural capsid proteins. The basis for the production of rAAV vectors is the fact that *rep* and *cap* genes can be deleted from the viral genome and provided in *trans*, making room to insert a transgene with transcriptional control elements, flanked by the viral ITRs [102]. There are inherent limitations of packaging genes into AAV due to its small size. However, extensive AAV research has determined ways to bypass this limitation by using a dual vector system that can expand the rAAV packaging capacity [103]. Vectors have also been created that offer a rapid onset of transduction for more efficient gene expression by circumventing the rate-limiting requirement for double-stranded DNA conversion but this does limit the transgene capacity [104].

Much of the knowledge surrounding the AAV life cycle has been discovered *in vitro*. It is thus possible that some of these processes do not occur in natural isolates. Ambiguity also arises due to the extraordinary promiscuity of the virus and its' ability to infect a wide range of cell types. Most of what is known is based on AAV2, the best-studied serotype, and may not be relevant to other serotypes. Only in-depth characterization of each serotype will result in a full understanding of relevant host factors and pathways involved in AAV infection.

AAV attaches to its target cell through a variety of receptors. Glycan recognition for cell entry has been studied for several AAV serotypes and can be separated into three major groups:

those that bind sialic acid (AAV1, -4, -5 and -6), those that bind heparin sulfate (AAV2, -3 and -6) and those that bind galactose (AAV9) [105]. For AAV2, heparin sulfate was first identified as the receptor *in vitro* although viral isolates from human tonsils do not bind as they lack two essential amino acids from the binding site [106, 107]. It has also been demonstrated that AAV2 can bind to fibroblast growth factor receptor 1 (FGFR1) [108]. While glycan receptors have not been identified for AAV 7 and -8, the 37/67-kD laminin receptor (LamR) has been identified as playing a role in cellular recognition for AAV8, a serotype isolated from rhesus macaques [109]. This receptor also plays a role in transduction with AAV2, -3, and -9. While several receptors have been identified, little is known about the particular signaling pathways and trafficking that occur after receptor binding.

It is thought that AAV2 enters the cell via clathrin-coated vesicles [110] and researchers currently hypothesize that it is trafficked to the nucleus via endosomes [111]. AAV replication initiates in the nucleus although how and where the DNA is released from the virion is unknown [112]. Inside the nucleus, helper virus functions are essential for a productive infection. If these factors are absent, limited expression of Rep68 and -78 occur leading to repressed AAV gene expression, inhibition of DNA replication and integration of AAV DNA into the host genome [113]. Helper virus co-infection can rescue the AAV genome in a latently infected cell.

Productive replication of AAV can be divided into three major steps: the single-stranded DNA genome must be turned into a double-stranded template for transcription, unidirectional strand-displacement replication occurs, and it is completed with terminal resolution of the self-annealed ITR. *Rep* genes are the first to be transcribed as Rep is involved in both negative (in the absence of helper proteins) and positive regulation of transcription [113]. Co-infection with Ad or HSV activates p5 and p9 promoters in order to transcribe Rep68 and -78 along with Rep 52 and -40 [114-117]. Following transcription of *rep* genes, extensive DNA replication occurs of which Rep is essential [118].



The general model for AAV DNA replication consists of unidirectional strand-displacement replication where the ITRs serve as the viral origin of replication, containing the Rep binding site (RBS) and terminal resolution site (TRS) [119, 120]. DNA replication starts with the ITR's self-annealing and providing a base-paired 3' hydroxyl group for unidirectional DNA synthesis. This is believed to be mediated by host replication machinery including polymerase  $\delta$  [121], although some components of the replication machinery may be provided by the helper virus. Once the AAV template has been copied, terminal resolution, via Rep, occurs in order to replicate the self-annealed ITR. The 3' hydroxyl end is regenerated by a strand-specific nick at the TRS which then provides the necessary hydroxyl group for replication through the viral ITR [122]. This replication cycle results in two possible products: double-stranded full length AAV and single-stranded full-length AAV displacement product. Currently, it is unknown whether either of these products can serve as templates for downstream events.

### **Viral helper and cellular proteins necessary for AAV replication**

Productive AAV infection requires helper functions that can be supplied by co-infection with helper viruses such as Ad and HSV. From Ad, helper functions are provided by E1a, E1b55K, E2a and E4orf6 along with viral associated RNA (VAI RNA). E1a activates other Ad promoters and relieves repression for the AAV p5 promoter [123]. The product of the E2a gene, a single-strand DNA binding protein (DBP) stimulates processivity of AAV replication *in vitro* [124] while E1b55K and E4orf6 cooperate to promote AAV replication and second-strand synthesis [125, 126]. These proteins also function as a ubiquitin ligase and degrade targets such as DNA repair proteins that limit AAV transduction [127]. VAI RNA stimulates expression of AAV proteins, most likely by preventing phosphorylation of EIF2alpha translation factor [128]. All of these Ad helper proteins work to alter the cellular environment to promote and enhance AAV replication.

The helper proteins that can be provided by HSV-1 are defined as HSV-1 replication proteins: the helicase/primase complex (UL5, UL8, and UL52) and the DBP ICP8 that is encoded by UL29 [129]. These are only the minimal proteins needed to replicate AAV but other HSV-1 proteins are capable of enhancing AAV replication. This includes the ICPO transactivator that activates *rep* gene expression [130] and the DNA polymerase complex (polymerase UL30 and its cofactor UL42) can contribute to efficient replication [131].

It has been demonstrated that AAV can replicate *in vitro* if purified Rep proteins are reconstituted with the following cellular enzymes: DNA polymerase  $\delta$ , proliferating cell nuclear antigen (PCNA), replication factor C (RFC) and the minichromosome maintenance complex (MCM) [121]. This replication is enhanced by the addition of DBPs from the cell such as RPA [124]. Cellular proteins that have been shown to bind to Rep or ITR are either required for replication or regulate aspects of the viral life cycle. Biochemical methods, genetic screens and cell-based assays have identified that importin alpha receptor which mediates nuclear import [132], Sp1 that mediates transcriptional activation [133] and the high mobility group protein 1 (HMG1) all interact and affect Rep functions [134]. Cellular factors that have been shown to bind to AAV ITR to regulate transcription include the chaperone-associated protein FKBP52 [135], cellular-zinc finger protein ZF5 [136] and human APOBEC3A [137]. These cellular proteins can function as negative regulators as ZF5 and FKBP52 block second-strand synthesis. Both cellular and viral factors help to regulate the temporal and spatial requirements of AAV DNA replication, capsid assembly and genome packaging with AAV replication taking place in the nucleus, viral DNA co-localizing at viral replication centers [138, 139] and cap proteins passing through the nucleoli to accumulate with Rep in the cytoplasm.

### **Gene expression and Rep proteins**

There are four Rep proteins: Rep78, -68, -52 and -40. In general, Rep52 and -40 are required for the accumulation and packaging of single-stranded genomes [140, 141] while Rep78 and -68, are involved in almost every step in the viral life cycle including: DNA replication [118, 142], site-specific integration [143-145], rescue of integrated genomes [146] and regulation of viral and cellular promoters [113, 114, 117, 142, 147-149]. Consistent with their multifunctional role in the life cycle, the Rep proteins possess a variety of biochemical functions with three of the major functional domains being present on Rep78, the largest Rep protein. The amino terminus is able to specifically bind DNA and has endonuclease activity while the central domain contains a nuclear localization sequence and motifs necessary for ATPase and helicase activity [150, 151]. Rep 78 carboxy-terminal domain contains a Zn-finger that has been shown to interact with many different cellular factors. The remaining Rep isoforms, Rep68, -52 and -40 have a combination of these functional domains which arises from alternative splicing and differential promoter usage within the *rep* open reading frame. All four isoforms possess helicase activity and this domain is highly conserved among parvoviral nonstructural proteins [150]. Despite the number of biochemical studies and characterization that has occurred, the molecular mechanisms underlying the biological functions carried out by Rep proteins are still not fully understood.

### **Genome integration and safety**

rAAV vectors are promising vectors for gene therapy and vaccines since they are capable of mediating long-term gene expression *in vivo*. However, serious safety concerns have arisen due to the ability of WT AAV to integrate into the transduced cell genome which could lead to the upregulation of genes and promote tumor formation. A significant amount of research has thus been undertaken to understand the integration mechanism and biological consequences of AAV integration.

The molecular mechanism of AAV integration was initially characterized *in vitro* using WT AAV2. It was found that viral-cellular junctions consistently mapped to one region in the human genome, chromosome 19q13.42, now referred to as AAVS1 [152, 153]. Further research demonstrated that recombinant viruses lacking the *rep* genes randomly integrated, suggesting that Rep is responsible for site-specific integration [154]. By supplying the large Rep proteins *in trans*, the site-specific integration into AAVS1 was restored and further evidence was provided when functional RBS and TRS motifs located within AAVS1 were found to be necessary and sufficient for site-specific integration [144, 145, 155]. However, the presence of alternative integration sites has been postulated and using a linker-selection mediated (LSM) PCR to enrich for AAV-chromosome fusion sequences, AAV integration sites were found throughout the entire genome, with only 10% of total events occurring in AAVS1. This suggested that targeted AAV integration is not as specific for AAVS1 as previously hypothesized [156]. Other hotspots for integration were found near consensus RBS sequences and included chromosome 5p13.3 denoted AAVS2 and on chromosome 3p24.3 denoted AAVS3. It was then proposed that when helper proteins are not present, Rep targets the AAV genome for integration near consensus RBS sites by initiating replication at this location [143, 156]. Once the DNA replication is initiated, strand-switching between the chromosome and viral DNA template allows for the incorporation of multiple copies of AAV genome into the locus. However, this mechanism remains hypothetical.

A mouse ortholog to AAVS1 was discovered and allowed for *in vivo* functional studies to be carried out [157]. Utilizing this system, it was shown that targeted integration of a marker gene resulted in strong transgene expression that persisted through differentiation of cells into multiple lineages [158]. When this occurred in blastocytes, site-specifically integrated cells were found throughout all tissues in the absence of a discernible phenotype in the animals, suggesting that AAVS1-targeted gene integration could result in the safe genetic modification of target cells [158]. Further studies using human embryonic stem cells identified that the initial Rep-mediated

integration involved a partial duplication of the target sequence, suggesting an AAV evolved mechanism for targeted gene addition. To date, it is still unknown whether integration is a significant component of the viral life cycle *in vivo* since most studies have been conducted *in vitro*.

While it is known that the preferred integration site for WT AAV2 *in vitro* is near RBS hotspots, the location and frequency of integration has not been demonstrated *in vivo*. Schnepf *et al* tested 175 tissue samples for the presence of AAV DNA. Only 9 samples were positive for the presence of AAV and using linear amplification-mediated-PCR (LAM-PCR), it was determined that the majority of AAV genomes were primarily arranged in a head-to-tail array with extensive deletions and rearrangements in the ITRs [159]. Only one AAV-cellular junction was identified and it was mapped to a highly repetitive satellite DNA element on chromosome 1. Due to the low frequency of AAV integration that was found, it was hypothesized that a majority of AAV genomes exist extrachromosomally. Using a linear rolling-circle amplification assay, it was demonstrated that most of WT AAV DNA exists as circular double-stranded episomes in human tissue [159].

rAAV vectors for therapeutics lack the *rep* gene which codes for the proteins responsible for integration thus theoretically, rAAV vectors should rarely integrate. However, it is still presumed that such vectors integrate to some degree. To determine the integration rate of an rAAV therapeutic vector, muscle and liver samples were taken from mice and patients that received an intramuscular injection of the licensed AAV1-LPL<sup>S447X</sup> vector [160]. It was found that the majority of AAV existed as concatameric rearrangements with deleted ITRS. In contrast to the *in vitro* data, no preferential vector integration within genes, CpG islands, palindromic sequences or ribosomal DNA loci were found. Instead, AAV was found integrated into random nuclear and mitochondrial hotspots. While integration was found in patients *in vivo* presenting a potential safety issue, the integration frequency of AAV is still well below that of integration-

competent retroviral vectors, suggesting that the use of AAV for therapeutics is safe [160]. Research has demonstrated that the integration frequency of AAV, in general, is higher in liver transductions compared to muscle [161-163]. A study investigating the genetic fate of rAAV genomes in mouse muscle demonstrating that >99.5% of vector DNA did not integrate and instead, existed as transcriptionally active monomeric and concatameric episomes [164]. This was confirmed in primates where rAAV integrated inefficiently but predominantly resided extrachromasomally, with gene expression persisting for years due to its bona fide chromatin structure [165].

However, even a low frequency of integration can still lead to potential genotoxicity [166]. Mice transduced with a high-dose, liver-directed, AAV-mediated gene transfer did not show a significantly higher rate of hepatocellular carcinomas 18 months post-transduction when compared to the control mice [167]. While integration was detected in active genes, CpG islands and G/C rich regions, gene expression data demonstrated that genes located near the integration sites did not show significant changes in gene expression pattern when compared to genes that were more distal to integration sites. This suggested that AAV integration into active genes did not upregulate gene expression and that there was no significant association between increased oncogene expression and AAV transduction [167]. While this study did support the safety of rAAV vectors, this will continue to be an active area of study, especially as the increase in AAV technology continues.

### **Epidemiology and consequences of pre-existing immunity to AAV serotypes**

For the universal application of AAV-mediated therapeutics, the presence of neutralizing anti-AAV antibodies is extremely problematic. Both pre-existing and acquired antibodies generated as a result of therapeutic use of rAAVs can inhibit the efficacy of this technology, especially if it requires repeated vector administration. Approximately 80% of the population is

seropositive for anti-AAV antibodies against serotypes 1, 2, 3 and 5 [168]. AAV2 is the most common human serotype and in several epidemiological studies, it was shown that 67% of the studied population had preexisting IgG antibodies [168, 169]. In all geographic regions studied, neutralizing antibodies (nAb) to AAV2 are the most prevalent, followed by antibodies to AAV1. Even binding antibodies that are incapable of neutralizing virus can trigger vector clearance by the immune system [170]. It has been demonstrated that in NHP with pre-existing immunity against AAV8, vector particles fail to reach the liver and instead accumulate in the spleen [171]. Unfortunately, individuals become seropositive for AAV quite quickly. It is most desirable to administer gene therapy shortly after birth or in early childhood however, anti-AAV antibodies can be detected at birth, suggesting vertical transmission of maternal antibodies and after birth, antibody levels, specifically IgG, increase after the first year of life [172]. While a majority of these studies detected antibodies by ELISA, which does not distinguish between neutralizing and non-neutralizing antibodies, high levels of IgG1, IgG2 and IgM correlate with nAb titers thus a certain estimation of neutralizing activity can be extrapolated [168, 173].

The high frequency of seropositivity within the human population has necessitated the use of alternative serotypes. While AAV1 and -2 are the most common human serotypes, there are AAV serotypes that are not as prevalent, such as AAV5, -6, -8 and -9. Of these, neutralizing factors to AAV8 and -9, are much lower compared to other vector types, with AAV8 being initially isolated from rhesus monkeys [168, 169, 174]. While the prevalence of nAb to AAV8 is lower compared to other serotypes, 19% of a sample population had low titers of nAb with an additional 38% positive for total IgG by ELISA [168]. Data indicates that even low levels of nAb can completely prevent transduction [171]. In addition, there is high sequence and structural homology among AAVs, differing by less than 15%, with some serotypes differing by only one amino acid [174]. The presence of AAV-reactive antibodies in a large proportion of the population can make it difficult to circumvent the detrimental effects of preexisting immunity

with the use of any AAV vector. For all serotype combinations, crossreactivity exceeds 50% [168]. Successful serotype switching not only relies on the lack of cross-reactivity between AAV variants but also depends upon the alternate AAV serotype having a similar tissue tropism. Due to the use of different receptors, AAV serotypes have different affinities for certain types of tissues. Thus while alternative serotypes may be utilized, there are still other challenges to overcome.

To bypass potential cross-neutralization activity between AAV serotypes, there has been a renewed effort in developing new AAV variants that show increased resistance to nAbs. Neutralizing epitopes have been identified for AAV2 [175] and AAV8 [99] and some approaches to design new serotypes alter the known antigenic regions of a particular serotype. For situations where multiple injections of the AAV vector would be required, to prevent the acquisition of immunity to the vector, it has been demonstrated that deleting CpG motifs which are recognized by TLR9, permits vectors to evade the adaptive immune response and establish prolonged transgene expression [176]. Several other approaches utilize an approach coined directed evolution where error-prone PCR, DNA shuffling of capsid sequences or staggered extension PCR [177] create new variants resistant to neutralization by preexisting antibodies [170, 178]. While these attempts at capsid engineering have been successful in isolating new AAV variants, none of these variants are completely resistant to nAbs and their infectivity and tissue tropism can be affected with the accumulation of various mutations [178].

Other strategies to minimize the effect of nAbs on transduction with rAAV have utilized plasmapheresis to decrease the total number of nAbs present in the blood [179, 180], immunosuppression [181, 182] or direct injection of the vector into the target tissue [183]. Unfortunately, while plasmapheresis and immunosuppression may be useful for gene therapy, it is not feasible for use in global vaccination as it can only be used on an individual basis. However, direct injection of the vector to the target tissue could reduce the effects of preexisting



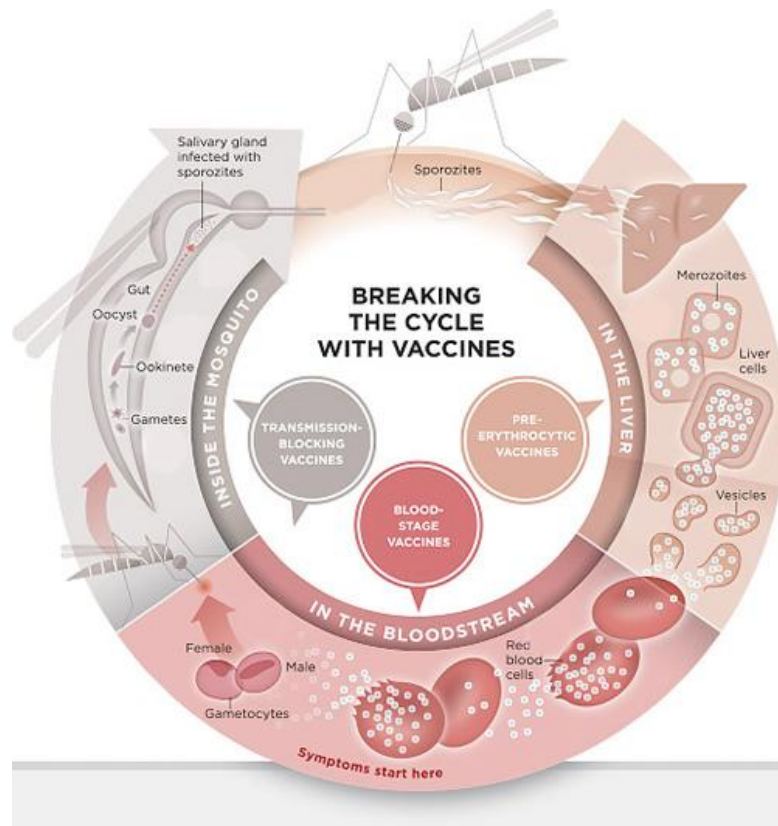
immunity in AAV-mediated vaccines as it limits contact of the vector with the circulatory system and thus, with nAb. It has become routine for many vaccines to be administered intramuscularly and, to avoid nAb, this is a very promising option.

Unfortunately, the presence of nAb is not the only preexisting immunity in a given individual, there is also an adaptive cell-mediated immune response. While the presence of nAb to AAV serotypes is more problematic than cell-mediated immunity as AAV vectors do not give rise to a strong cellular immune response against the vector [184], a weak immune response that is generated against AAV capsids can still be detrimental to transduction efficiency [185-187]. However, the presence of an AAV-specific T cell response does not always result in the ablation of transduced cells. All patients that received an rAAV.hAAT vector designed to treat  $\alpha 1$ -antitrypsin deficiency developed a detectable nAb and a cellular immune response as detected by interferon- $\gamma$  enzyme-linked immunospot responses to the capsid [188]. While there was even evidence of inflammatory cells in muscle biopsies close to the injection site, hAAT levels were elevated and sustained out to at least 90 days post-injection suggesting that the cellular immune response against the AAV capsid does not necessarily suppress transgene expression in every situation. Thus, the role that the cellular immune response will play on AAV-mediated transgene expression cannot easily be predicted. However it has been shown that a serotype's ability to bind heparin through the RXXR motif directly activates capsid-specific T cells [186]. Site-directed mutants of this motif and naturally lacking serotypes failed to activate a T cell response leading to prolonged transduction of cells. Thus, through the careful choice of AAV serotype, a capsid-specific T cell response leading to the loss of transgene expression through cell-mediated killing of transduced can be avoided.

### **rAAV vectors for vaccines and immunotherapy**

While the majority of rAAV vectors have been created to be used in gene therapy, the utility of this virus for vaccines was quickly realized, as it exhibits long-term transgene expression. For example, rAAV was used to express Nipah virus G protein and protect mice from challenge [189]. AAV vectors were also used for immunotherapy where a full-length monoclonal antibody was expressed *in vivo* instead of an antigen. An antibody heavy chain and light chain are linked by a self-processing peptide and furin cleavage site so as to self-assemble upon translation. Engineered immunity delivered this way has been accomplished and protective against HIV [190, 191], anthrax [192], respiratory syncytial virus (RSV) [193], simian immunodeficiency virus (SIV) [194], influenza [195] and cancer [196]. Vectors not only have been engineered to continually stably express monoclonal antibodies but also express antibodies only in the presence of a given inducer via a regulated promoter, allowing for regulated delivery of therapeutic monoclonal antibodies in the clinic [197].

The potential uses for AAV technology in the biomedical field is just being realized. With the first AAV gene therapy vector being licensed recently in Europe, the path has been paved for more AAV-mediated therapies to follow, especially as research continues to reinforce the safety of this vector. Importantly, AAV is now being utilized in the fairly new field of engineered immunity in order to deliver protective monoclonal antibodies *in vivo*. In particular, an AAV-vectored HIV monoclonal antibody, b12, is set to begin clinical trials shortly. Continued investigation and monitoring of AAV vector safety needs to occur in addition to further research on its clinical applications.



**Figure 1: Life cycle of the malaria parasite.**

Pre-erythrocytic vaccines targeting antigens expressed on sporozoites or infected hepatocytes can prevent both disease and transmission. Blood-stage vaccines targeting antigens expressed on merozoites and infected red blood cells work to reduce disease severity and transmission blocking vaccines targeting either gametocytes or developmental stages within the mosquito prevent transmission of the *Plasmodium* parasite. Adapted from [www.malariavaccine.org](http://www.malariavaccine.org).

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## **Chapter Six:**

### **Vectored antibody gene delivery protects mice against sporozoite challenge**

## Abstract

*Plasmodium* sporozoites can be neutralized *in vitro* by monoclonal antibodies (mAb) against the circumsporozoite protein (CSP). Passively transferred mAb against *P. falciparum* CSP can block liver invasion by sporozoites of a transgenic rodent parasite that expresses *P. falciparum* CSP (Pb-Pf), preventing infection in mice. A single un-neutralized sporozoite can initiate infection, necessitating sustained high-titer neutralizing antibodies for lasting protection. Despite this, attempts at targeting CSP for a vaccine have fallen short of expectations, in part due to inability to induce durable high-titer antibodies.

Recently, David Baltimore's laboratory developed an adeno-associated virus type 8 (AAV8) platform that efficiently delivers pre-formed mAb genes *in vivo* and directs sustained, high-level mAb production. In collaboration with the Baltimore laboratory, we have adopted that technology to express humanized mAbs against the central repeat region of the CSP protein of *P. falciparum* in mice. Mice developed high titer human IgG antibodies as early as 1 week post transduction and levels have remained constant for more than 28 weeks at 200 to 1000 µg of IgG/ml. Mice transduced with humanized CSP mAb 2A10 (2A10-AAV) and challenged intravenously with  $10^4$  Pb-Pf sporozoites, exhibited a statistically significant decrease in parasite liver burden. Furthermore, 2A10- and 2C11-AAV mice challenged by infected mosquito bite displayed a statistically significant delay in time to patency, with 70% and 30%, respectively, being sterilely protected. Examination of antibody levels in individual 2A10-AAV mice revealed that all mice with human IgG concentrations above 1mg/mL were completely protected. This suggests that exceeding this antibody threshold results in consistent sterile protection and establishes that vectored mAb gene delivery has the potential to be an effective form of malaria control.

## Introduction

Despite tremendous efforts at control and prevention, malaria continues to represent a major public health burden and results in approximately 219 million cases, with 660,000 deaths in children under 5 each year [1]. Among infectious diseases, malaria ranks fourth in causes of death. With emerging drug resistance among parasites, insecticide resistance among mosquito vectors and the political and economic difficulties associated with sustained traditional malaria control, efforts on the development of new approaches to malaria prevention are a high priority [2]. Although multiple approaches to inducing effective immunity by vaccines have been employed, only one has proven sufficient to elicit durable protection but is not clinically feasible for deployment in developing countries. The development of a vaccine against malaria is complicated by the complexity of the *Plasmodium falciparum* parasite life cycle and its unique potential target antigens for each of the developmental stages in the human host [3]. These stages include the sporozoites that are injected upon mosquito bite, infected hepatocytes, the merozoites that infect and rupture red blood cells, leading to the clinical disease, and the gametocytes which are infectious for *Anopheles* mosquitoes. This wealth of potential immune targets has led to a diversity of approaches to induce anti-malarial immunity, with research underway that focuses on each stage of the life cycle, employing diverse antigens and a wide variety of systems for antigen expression [3].

Among these potential strategies, a vaccine against sporozoites, the pre-erythrocytic stage, would be the most advantageous because of its potential to prevent infection completely, eliminating both pathology and transmission. This stage also represents a bottle-neck in the *Plasmodium* life cycle, with a few dozen to a few hundred sporozoites deposited in the skin upon mosquito bite [4, 5]. Protective immunity against sporozoites in humans has been achieved by the use of irradiated sporozoites however, this vaccine is not practical for global use, as protection requires individuals to be bitten by roughly 1000 irradiated infectious mosquitoes or receive 5 intravenous injections of irradiated sporozoites [6-9]. The

predominant immune response in immunization with radiation-attenuated sporozoites is elicited against the circumsporozoite protein (CSP) which coats the surface of sporozoites [10]. Importantly, it has been demonstrated that antibody targeting the central tetrapeptide (NANP) repeat region of CSP can completely protect from sporozoite infection in mice and monkeys by preventing sporozoite motility [11-13]. This immunodominant epitope is found in 30 or more consecutive copies and is stringently conserved in *P. falciparum* isolates throughout diverse geographical areas [14, 15]. The induction of CSP antibodies is a central goal of many pre-erythrocytic immunization efforts, including the most advanced malaria vaccine candidate RTS,S/AS01, a hepatitis B virus-like particle (VLP) vaccine that induces both antibody and cell-mediated responses to CSP in humans [16, 17]. While preliminary data from a phase 3 clinical trial indicated that this vaccine was 50% effective in preventing clinical and severe malaria over the course of a year post immunization in children 5 to 17 months of age, there was no detectable reduction in malaria deaths [17]. Results from this trial also suggested that vaccine-mediated protection wanes fairly rapidly, within 6 months post vaccination, and therefore, this vaccine is not suitable for deployment [18, 19]. The difficulty in developing a vaccine to induce protective immunity against CSP lies in the brief period of time that sporozoites are exposed to antibody before invasion of liver cells and that a single un-neutralized sporozoite is capable of initiating a full-blown infection [5, 20]. Thus, permanent, unnaturally high levels of CSP antibodies would be required for effective anti-malarial humoral immunity.

Adeno-associated virus (AAV) is a helper dependent virus of the parvoviridae family with an ~5kb single-stranded DNA genome flanked by inverted terminal repeats (ITRs). Productive AAV infection requires helper functions that can be supplied by either adenovirus or herpes simplex virus. AAV is ubiquitously prevalent in the population, with 60% of the human population having neutralizing antibodies to human serotypes by age 10 [21]. However this virus causes no known disease. Due to its ability to effectively transduce cells and exist primarily as an episome in the nucleus, recombinant AAV (rAAV) has been utilized as gene therapy vectors in 47 clinical trials with an exemplary safety record



[22]. The first AAV vectored gene therapy was approved in Europe in 2012 to treat lipoprotein lipase deficiency (LPLD) [23]. Due to preexisting immunity to common serotypes such as AAV2 [24, 25], recent research has looked at alternative serotypes with low prevalence such as rhesus monkey derived AAV8 [26]. This particular serotype also lacks heparin binding activity that has been shown to be associated with an increased T cell response against the vector, making this vector long-lived *in vivo* [27].

Antibody gene transfer involves the delivery of genes via an AAV8 vector that encodes previously isolated and characterized broadly neutralizing monoclonal human IgG antibodies *in vivo* resulting in durable, high level expression upon a single intramuscular injection, capable of protecting mice from HIV and influenza [28, 29]. This malleable system allows for the expression of new human IgG monoclonal antibodies by inserting the sequences of the monoclonal variable regions into this transgene (Figure 1). Termed vectored immunoprophylaxis (VIP), this approach utilizes a vector that was systematically optimized to improve transgene expression of a human IgG<sub>1</sub> mAb *in vitro* and *in vivo*. The VIP expression vector consists of AAV2 ITRs necessary for genome packaging and transcription of the transgene, a novel promoter that combines a cytomegalovirus (CMV) enhancer,  $\beta$ -actin promoter, and ubiquitin (UBC) enhancer region flanked by a splice donor and splice acceptor (Figure 1A), along with a human cDNA IgG<sub>1</sub> transgene, a woodchuck hepatitis posttranscriptional regulatory element (WPRE) for improved expression and an SV40 late-polyadenylation signal (Figure 1B). Antibody expression was improved by optimizing the IgG<sub>1</sub> transgene. Human growth hormone (HGH) derived signal peptides were fused to the antibody variable regions. The heavy and light chain genes were separated by an F2a self-processing peptide, mutated to better reflect mammalian codon usage with an incorporated furin cleavage site at the N-terminus for optimal processing; this allows for secretion of independent light and heavy chains. Predicted splice donor and acceptor sites were mutated in the constant regions to prevent potential inappropriate splicing of the transcript (Figure 1C). Importantly, this system allowed for swappable light and heavy chain variable regions by restriction enzyme digest of the modular VIP vector plasmid [28]. Thus, only the mAb sequence of the variable regions is needed to create a vector that

expresses humanized IgG<sub>1</sub> mAb *in vivo* upon a single intramuscular injection. This system has conferred highly effective protection against HIV in humanized mice and from influenza in both healthy and immunocompromised animals [28, 29], making it a powerful system for infectious diseases in which antibody alone is protective.

Here we describe a novel means of generating high protective levels of antibodies against the CSP of *P. falciparum* using the VIP system. We demonstrate that by expressing high levels of neutralizing CSP mAb, we are able to protect mice from stringent *P. falciparum* sporozoite challenges. Our results suggest that with successful translation to humans, this prophylactic approach could be capable of protecting humans from malaria.

## Materials and Methods

### *AAV virus production and functional validation*

To express b12, 2A10 and 2C11 antibodies in mice, the variable regions of these antibodies were synthesized from published sequences [30] and cloned into the VIP expression vector as previously described (Figure 1) [28, 29]. Purified AAV was quantified using qPCR as previously described [28, 31]. To validate the functional activity of each lot of virus, each stock was used to infect 293T cells and the concentration of antibody in the cell supernatant was measured. In a 12-well plate containing 500,000 cells per well,  $10^{11}$  genome copies (GC) of each virus were added per well. 6-days post infection, supernatants were removed and quantified for total human IgG production by ELISA [28]. This work was done in Dr. David Baltimore's laboratory at California Institute of Technology by Dr. Alex Balazs.

### *AAV intramuscular injection and serum collection*

Aliquots of previously titered viruses were thawed on ice and diluted in TFB2 (100mM sodium citrate, 10mM Tris, pH 8) to achieve the predetermined dose of  $1 \times 10^{11}$  genome copies in a 50  $\mu$ L volume. Inbred 5-to 8-week old C57BL/6 (NCI) female mice, in groups of five or ten, were given a single 50  $\mu$ L intramuscular (i.m.) injection into the cranial thigh muscle with a 29G needle. At various times after vector administration, blood was collected from the cheek vein into serum separator tubes (BD). Tubes were spun for 10 minutes at 5,000 RPM in order to separate sera from red blood cells. Sera was collected and stored at  $-80^{\circ}\text{C}$ .

For radiation-attenuated sporozoite immunization, five inbred 5-to-8 week old C57BL/6 female mice were vaccinated intravenously with  $1 \times 10^5$  to  $1.5 \times 10^5$  *Pb-Pf* sporozoites (see below) exposed to 20,000R in a  $\gamma$  irradiator. Mice received a total of three doses of irradiated sporozoites spaced apart by at least two weeks. Prior to each dose, mice were bled and anti-CSP antibody levels were measured.

### *Quantification of antibody production by ELISA*

For detection of total human IgG, ELISA plates were coated with 0.1 µg per well of goat anti-human IgG-Fc antibody (Bethyl) overnight [28]. Plates were washed three times with PBS containing 0.1% Tween20 (PBS-T) and three times with PBS followed by blocking with 1% BSA (Sigma) in PBS (PBS-1% BSA) for 1 hr. Samples were serially diluted three-fold in PBS-1% BSA starting at 1:1500 and incubated for 1 hr at room temperature (RT). Plates were washed as above and incubated with HRP-conjugated goat anti-human kappa light chain antibody (1:10,000; Bethyl) for 1 hr. Plates were washed a final time and samples were detected using ABTS Peroxidase Substrate System (KPL). A standard curve was generated using Human Reference Serum (Bethyl) and mouse prebleed sera was used to establish background. Plates were read using a Molecular Devices Emax microplate reader.

For detection of CSP antibodies, plates were coated with 0.05 µg per well of recombinant *P. falciparum* CSP purified from MR-272 plasmid (Malaria Reference and Research Resource MR-272) overnight [32]. All subsequent steps are described above except a standard curve was not utilized. Purified 2A10 was used as a positive control and the endpoint ELISA titer was determined as the highest dilution at which the optical density was two times greater than the background. For detection of CSP antibodies in irradiated sporozoite-immunized mice, an HRP-conjugated anti-mouse IgG (1:5000 dilution; GE Healthcare) was used as a secondary.

To detect anti-human Fc antibodies, plates were coated with 1 µg per well human IgG from the Human Reference Serum (Bethyl) overnight. All subsequent steps are described above except sample dilutions started at 1:100, plates were incubated with an HRP-conjugated anti-mouse IgG and a standard curve was not utilized. Anti-human kappa light chain antibody was used as a positive control. The endpoint ELISA titer was determined as the highest dilution at which the optical density was two times greater than background.

### *Parasites*

Transgenic murine *P. berghei* sporozoites expressing the human *P. falciparum* CSP repeat region (*Pb-Pf*) were used in all experiments. It has previously been demonstrated that antibodies specific to the repeat region of *P. falciparum* CSP are capable of recognizing and neutralizing *Pb-Pf* sporozoites [33].

#### *Immunofluorescent assay (IFA)*

To determine whether serum bound to sporozoites, slides (Tekdon Inc; Poly-L-Lysine coated) were coated with 10  $\mu$ L of *Pb-Pf* [32] at a concentration of  $4\text{--}6 \times 10^5$  sporozoites per mL and air-dried. Slides were blocked for 1 hr with 10  $\mu$ L of PBS-1% BSA. Serum samples from each mouse per group were pooled and 500ng of human IgG, determined from human IgG ELISA titers, were diluted in PBS-1% BSA and incubated on slides for 1 hr at RT. Slides were washed in PBS-1% BSA and 10  $\mu$ L of FITC-labeled goat anti-monkey IgG (H+L) (KPL) was added for 1 hr at RT. Prior to visualizing, slides were washed with PBS-1% BSA and coverslips were mounted with Prolong Gold antifade reagent with DAPI (Molecular Probes). Fluorescent sporozoites were visualized using an upright fluorescence microscope (Nikon Eclipse 90i).

#### *Sporozoite challenge.*

C57BL/6 mice were used since they have been shown to be highly susceptible to sporozoite challenge [34]. Infected *Anopheles stephensi* mosquitoes were dissected in IMDM media for isolation of salivary glands which were spun down at 7000 rpm for 1 minute at 4°C. Most of the supernatant was aspirated off the resulting pellet and this pellet was ground in  $\sim 100$   $\mu$ L of media to break open the salivary glands. This suspension was spun at 1000rpm for 1 minute at 4°C to collect sporozoites present in the supernatant which were counted using a hemocytometer. Mice were challenged intravenously with  $1.0 \times 10^4$  to  $2.0 \times 10^4$  transgenic *Pb-Pf* parasites [33]. Approximately 40 hours later, mice were euthanized to assess parasite burden in livers. Whole livers were homogenized in denaturing solution and RNA was

extracted as previously described [35, 36]. After cDNA synthesis, parasite loads were determined by quantitative PCR for *P. berghei* 18S rRNA [37] and mouse GAPDH was used as an internal control.

For the assessment of sterile protection, *Anopheles stephensi* mosquitoes infected with *Pb-Pf* parasites were starved overnight. Mice were anesthetized with 300 to 350  $\mu$ L of 2% Avertin and were subjected to feeding from 10 or 15 mosquitoes for 5 minutes. The number of mosquitoes that fed on each mouse, as indicated by the presence of blood in the midgut, was recorded. Starting on day 4, blood smears were made daily until day 14 and observed under a microscope for blood stage parasites. Smears were fixed with methanol for 30 seconds before staining with a 10% Giemsa stain solution (Sigma) for 15 minutes. The day post infection that blood stage parasitemia was evident was recorded as day of patency. After confirmation of parasitemia, mice were euthanized. Mice were considered to be sterilely protected if there was no evidence of parasitemia by day 14.

#### *Statistical analysis*

The majority of data and statistical analysis were performed using Prism software (GraphPad) using a Kruskal-Wallis test. Differences were found to be significant when p was less than 0.05 (\*) or 0.01 (\*\*). Kaplan-Meier day to patency survival curves were analyzed using a logrank test.

## Results

### *Characterization of VIP vectors expressing mAb against P. falciparum CSP in vitro and in vivo*

Current vaccination strategies have not resulted in an effective malaria vaccine despite decades of effort. It is known that CSP antibodies alone can neutralize and prevent infection by sporozoites, but this requires very high sustained levels [12, 13]. Recently, it has been demonstrated that genes encoding HIV-neutralizing antibodies delivered to mice by a newly-designed AAV8 vector can direct long-lived high-level mAb production (Figure 1) [28]. These mAbs protected humanized mice from high doses of HIV. This approach, termed vectored immunoprophylaxis (VIP), bypasses the uncertain pathway of immune induction by antigen and is potentially applicable to any disease against which an antibody response alone is protective [28, 29]. Due to the existence of protective CSP antibodies and given the long-lived nature of VIP-directed mAb expression, it seemed plausible that the VIP approach could be used against malaria.

In collaboration with Dr. David Baltimore, AAV vectors with the capsid from serotype 8 were made that expressed the sporozoite humanized IgG<sub>1</sub> mAbs 2A10 or 2C11, both directed against the repeat region of CSP. 2A10 and 2C11 arose independently from mice immunized with *P. falciparum* sporozoites and completely block sporozoites attachment and invasion of hepatic cells *in vitro*. The AAV vectors expressing CSP mAb were characterized *in vitro* for human IgG antibody expression in the supernatant of infected 293T cells (Figure 2). Antibody was expressed to high titer six days after infection and notably, 2A10- and 2C11-AAV vectors produced similar levels of human IgG antibody as b12-AAV, a previously characterized antibody expression VIP vector control [28].

To determine expression by these VIP vectors *in vivo*, C57BL/6 mice were injected i.m. with  $1 \times 10^{11}$  GC of 2A10-AAV, 2C11-AAV, b12-AAV or media alone. Within one week post transduction, all AAV-transduced mice expressed between 50-1000  $\mu\text{g/mL}$  of human IgG antibodies. Expression continued to increase to 4 weeks post administration and was sustained out to 8 weeks (Figure 3A). To

ensure that the initial manipulation of the CSP-specific mAb did not affect their ability to bind to CSP, sera from transduced mice were used to probe recombinant CSP by ELISA (Figure 3B) and whole *P. berghei* sporozoites expressing the *P. falciparum* repeat region (*Pb-Pf*, Figure 3C) by immunofluorescence. 2A10- and 2C11-transduced mice expressed antibodies that bound to recombinant CSP with similar kinetics as human IgG antibody expression (Figure 3B). Transduced mouse sera also were able to recognize whole sporozoites by immunofluorescence (Figure 3C). Sera from b12-AAV mice did not recognize recombinant CSP or *Pb-Pf* sporozoites, demonstrating the specificity of the 2A10 and 2C11 antibodies.

#### *Intravenous challenge of AAV-transduced mice with Pb-Pf sporozoites*

To test the ability of VIP to protect mice from a rigorous sporozoite challenge *in vivo*,  $1.0 \times 10^4$ - $2.0 \times 10^4$  *Pb-Pf* sporozoites isolated from infected *Anopheles stephensi* mosquitoes were injected intravenously (i.v.) into mice eight weeks post transduction with 2A10-, 2C11- or b12-AAV vectors. Antibodies to CSP block invasion of liver cells therefore, mice were sacrificed 40-42 hours post infection, a time when viable sporozoites will have successfully invaded and replicated in liver cells. RNA was extracted from liver homogenates to quantitate, via qPCR, the *P. berghei* 18S rRNA copies in the liver to reflect the parasite burden [37]. In two independent experiments, 2A10-AAV mice had a statistically significant reduction in parasite burden as compared to b12-AAV mice (Figure 4A). Notably, 7 2A10-AAV mice had 18S rRNA levels that were indistinguishable from levels in uninfected mice. A statistically significant reduction in parasite burden was achieved with the 2C11-AAV mice in one challenge (data not shown).

It is reasonable to hypothesize that the varying parasite burden within the 2A10-AAV mice is due to variable human IgG antibody concentrations. To determine if there is a correlation between antibody concentration and liver parasite burden, the human IgG antibody concentration immediately prior to challenge was graphed against the *Pb-Pf* 18S rRNA copies in the liver for each 2A10-AAV transduced



mouse (Figure 4B). While there was no statistically significant correlation ( $p=0.9640$ ,  $r=0.03030$ ; Figure 4B), there was a trend where mice with higher human IgG antibody titers exhibited lower parasite burden.

#### *Challenge of VIP-transduced mice with Pb-Pf sporozoites by infected mosquito bite*

While an i.v. sporozoite challenge is advantageous to control for the number of sporozoites each mouse receives, it is an unnatural route of infection and sporozoite viability is variable. A mosquito bite challenge utilizes the natural route of infection and allows for determination of sterile protection. However, the number of sporozoites per mouse is variable. To assess the protective capacity of VIP via a more natural route of infection, in two separate experiments, mice were exposed to the bites of 10 *Pb-Pf* infected *A. stephensi* mosquitoes for 5 minutes 11 weeks after transduction. Mice that received three doses of  $1 \times 10^5$  to  $1.5 \times 10^5$  irradiated *Pb-Pf* sporozoites were included as a positive protection control, as irradiated sporozoites are the gold standard for malaria vaccines. Since there is a potential correlation between human IgG antibody concentration and protection, mice were bled prior to challenge to quantitate their individual antibody levels.

In a mosquito bite challenge, mice can be partially protected, as indicated by a statistically significant delay in time to patency where a single day delay in patency represents 90% of the sporozoites being neutralized [38], or mice can be sterilely protected through neutralization of all infectious sporozoites. Therefore, starting at 4 days post infection, mice were bled daily and blood smears were visually assessed for blood-stage parasitemia. The presence of parasitemia indicates that sporozoites escaped anti-CSP mAb and successfully invaded the liver where they replicated and differentiated into merozoites in order to invade red blood cells. Immunization with 2A10- and 2C11-AAV vectors produced a statistically significant delay in time to parasitemia (Figure 5C). In addition, 60% of 2A10- and 30% of 2C11-AAV transduced mice were sterilely protected. B12-AAV mice and mice that only received media all exhibited blood stage parasitemia by day 6 while irradiated sporozoite vaccinated mice were completely protected from challenge (Figure 5C).

While there was a trend associated with reduced liver burden and mouse antibody concentration in the i.v. challenge, another layer of complexity was added to the mosquito bite challenge with each mouse receiving a variable number of sporozoites. To investigate potential correlations of protection with antibody concentration or number of blood-fed mosquitoes, the human IgG antibody concentration was measured immediately prior to challenge. Both the number of blood-fed mosquitoes and human IgG concentration were graphed against the day to patency to detect any protective correlations. All AAV transduced mice exhibited 100-1000  $\mu\text{g/mL}$  of human IgG (Figure 5A), with 2A10- and 2C11-AAV having high titers of anti-CSP antibodies (Figure 5B). Mice that received irradiated sporozoites did not have any human IgG but did have CSP-specific antibodies that were a log lower in titer compared to 2A10- and 2C11-AAV. However, irradiated sporozoite vaccinated mice, unlike AAV-transduced mice, do not solely have a humoral response against CSP but also have a T cell response to offer protection against sporozoite challenge. A statistically significant correlation was seen between antibody concentration and protection for 2A10-AAV mice ( $p=0.0105$ ,  $r=0.7814$ ; Figure 6A). However, this was not the case in 2C11-AAV mice ( $p=0.2058$ ,  $r=0.4377$ ; Figure 6B). Although the number of mosquito bites per mouse varied, thus resulting in mice inoculated with different numbers of parasites, this did not correlate with day to patency in either 2A10- ( $p=0.5042$ ,  $r=0.3022$ ; Figure 6C) or 2C11-AAV mice ( $p=0.9460$ ,  $r=0.01573$ ; Figure 6D).

#### *Dose-response of AAV vector in mice*

To determine the effect of vector dose on antibody concentration and protection from sporozoite challenge, mice were transduced with decreasing doses of the 2A10-AAV vector. Antibody expression measured over time revealed a clear dose-dependent expression at all time points analyzed (Figure 7A). All mice were challenged, via infected mosquito bite, at eleven weeks post transduction and were monitored for infection by blood smear. While all b12-AAV mice reached patency by day 6, 2A10-AAV mice exhibited a dose-dependent protection response with the lowest AAV dose,  $3 \times 10^9$  GC, reaching

patency by day 7. Sterile protection was achieved in 10 percent of the  $1 \times 10^9$  GC mice, 40 percent of the  $3 \times 10^9$  GC mice and 70 percent of the highest AAV dose,  $1 \times 10^{11}$  GC (Figure 7B). The correlation between 2A10 antibody concentration and day to patency was statistically significant ( $p=0.0013$ ,  $r=0.4424$ ; Figure 7C), while the number of mosquito bites did not impact the day to patency or sterile protection ( $p=0.2020$ ,  $r=-0.1835$ ; Figure 7D). Notably, all mice that had greater than 1mg/mL of 2A10 were completely protected from sporozoite challenge.

#### *Evaluation of VIP expression of human IgG over time*

In order to combat malaria, a vaccine must provide protection for an extended period of time. To determine the longevity of the expression of human IgG by AAV transduction, antibody levels in transduced mice were monitored over 36 weeks. The serum antibody concentration plateaus around eight weeks post-transduction and was maintained for the duration of the 36-week study (Figure 8). This demonstrates the utility of VIP to stably express protective antibodies for long periods of time, suggesting the feasibility of using this technology for effective malaria control.

#### *Anti-human Fc immune responses*

Viral-vectored gene therapy treatments may induce the development of an immune response against the transgene product which could negatively impact the efficacy of the therapy [39-43]. To determine if transduced mice were generating a detectable immune response against the AAV-encoded mAb transgene, an ELISA was developed to test for the presence of mouse antibodies that recognize the human Fc region. In agreement with previous studies of VIP and AAV-induced tolerance in mice, fewer than half of the mice developed a detectable immune response against the human Fc (Figure 9B). While some animals generated antibodies against the transgene, these did not appear to impact human IgG levels or protection from sporozoite challenge (Figure 4A and 7C). However, there was an increase in concentration and frequency of anti-transgene antibodies in transduced mice over time (Figure 9A).

Although ELISA data suggests that most transduced mice do not develop an immune response against the human transgene, it is possible that anti-human Fc antibodies exist but are not being detected as they are tied up in immune complexes (IC) that have been deposited in tissue [44]. To further investigate potential deleterious effects due to high expression of human IgG, a group of five mice were transduced with 2A10-AAV while another group only received media. At 26 weeks post transduction, both groups of mice were euthanized and assessed for any gross pathological differences between the transduced and non-transduced mice. There was no pathological evidence of IC deposition in any tissue, with particular attention paid to the kidney glomeruli, suggesting that an immune response against the human Fc is minimal. Additionally, there were no consistent pathological effects attributed to transduction or high sustained expression of human IgG (data not shown)

## Discussion

Conventional vaccination strategies based upon exposure to an antigen have not resulted in an effective malaria vaccine. The most advanced conventional vaccine candidate, RTS/S, comprised of hepatitis B VLPs expressing the carboxy-terminal half of CSP, has resulted in insufficient protection that rapidly wanes with time [16, 17]. Even naturally acquired immunity develops slowly and does not yield in sterile protection [3]. The only strategy that consistently provides sterile protection is vaccination with radiation-attenuated sporozoites, which is not clinically feasible in areas of high malaria burden as it requires bites by multiple infected irradiated mosquitoes or at least five intravenous injections with irradiated sporozoites [6-8]. Therefore, a different approach needs to be taken in the development of vaccines against malaria.

It has been known for decades that antibodies targeting the repeat region of CSP can prevent malaria infection by inhibiting sporozoites from invading liver cells [12, 13]. Even though immunity to CSP targets a bottle-neck phase in the malaria life cycle, achieving high enough antibody titers to neutralize all sporozoites prior to reaching the liver has been difficult as liver invasion by one parasite results in clinical infection. Dr. David Baltimore's laboratory at California Institute of Technology recently demonstrated a way to express high titer monoclonal human IgG antibodies *in vivo* by using an AAV vector (Figure 1). This system was capable of protecting humanized mice from an HIV challenge and immune-deficient mice from a lethal influenza challenge, demonstrating the utility of this technology in protecting against infectious diseases where antibody alone is protective [28, 29]. To test whether this approach would work for malaria, we collaborated with the Baltimore laboratory to create and characterize AAV2/8 vectors expressing humanized monoclonal CSP antibodies 2A10 or 2C11 [30]. These vectors directed mAb expression at ~100-1000 µg/mL of human IgG, as early as one week post transduction, *in vitro* (Figure 2) and *in vivo* (Figure 3, 5, 7, 8). Expressed mAbs recognized both recombinant CSP and *Pb-Pf* sporozoites (Figure 3B, C) and led to a statistically significant reduction in

liver parasite burden following i.v. challenge (Figure 4). When challenged by infected mosquito bite, a more natural route of infection, there was a statistically significant delay to patency in mice, with a subset that were completely protected (Figure 5, 7). The expression of these CSP-specific mAb by AAV vectors was sustained out to at least 36 weeks (Figure 8), with less than half of the mice exhibiting a detectable anti-transgene immune response (Figure 9). Detectable antibodies against the human Fc did not affect the human IgG antibody levels or ability of mice to be protected from sporozoite challenge. VIP thus demonstrates potential for use as a novel malaria prevention strategy.

The protection of 2A10-AAV mice from sporozoite challenge was dependent upon antibody concentration (Figure 6A, 8C). All mice with greater than 1mg/mL CSP-specific human IgG were completely protected (Figure 8C). It is unknown if this high concentration is needed to evenly distribute antibody throughout the circulatory system or is necessary to allow for multiple antibodies to bind to a single sporozoite to inhibit mobility. Additionally, the antibody threshold needed to consistently provide protection in humans may differ. Human antibodies have a significantly longer serum half-life in humans than in mice, which may result in higher steady-state levels than were obtained in this study [45, 46].

2A10 and 2C11 antibodies are protective mAbs with known sequences that both target the CSP NANP repeat and are routinely used by collaborators [30], making them ideal for use in the VIP system. An inherent problem in utilizing mAb to one target antigen is the potential for immune escape through mutations. It is unlikely that CSP repeat region escape mutants will emerge, as it would require simultaneous mutation of 30 to 50 NANP and NVDP repeats to prevent antibody binding [12, 47, 48]. While 2A10 is very well characterized and has a high affinity for the CSP tetrapeptide repeat, there are other CSP mAb with a greater protective capacity that target either the repeat regions or other domains (Zavala, unpublished). By making VIP vectors expressing these antibodies, we could continue to optimize this technology and improve protection in mice and Aotus monkeys, with the eventual goal of entering clinical trials. Additionally, AAV vectors could express mAb that target other sporozoite

antigens or antigens present in different stages of the parasite. While this particular study only utilized mAb targeting the repeat region of CSP, multiple stages of the parasite could easily be targeted at once via different mAb. There is limitless potential for this technology and multiple approaches to be taken to continue to optimize it as a form of malaria control.

The induction of antibodies from natural exposure to AAV early in life can compromise the use of AAV as a gene therapy or VIP vector by preventing efficient transduction. Approximately 30-60% of the population has neutralizing antibodies (nAb) specific for AAV2 and AAV1, the most common human serotypes [49]. Due to the prevalence of nAb to human serotypes, AAV8, isolated from rhesus macaques [26], was used in VIP vectors since it was anticipated to have a much lower seroprevalence among humans. However, in a worldwide epidemiological study of nAb to AAV serotypes in human sera, a percentage of the population, ranging from approximately 15-30%, are seroprevalent for anti-AAV8 nAb at serum dilutions of >1:20 [49]. The frequency of binding but non-neutralizing antibodies that could aid in opsonization of AAV particles is even higher with 38% seropositivity for AAV8 [25]. While there is still a significant advantage of using AAV8 over AAV1 or AAV2 in overcoming preexisting immunity in humans, it should be noted that there was a higher prevalence for AAV8 nAb in Africa, a target population for the VIP-mediated malaria control. Even very low detectable nAbs could be problematic in a large population.

While the low prevalence of anti-AAV8 nAb may present difficulties in administering VIP for malaria control, there are multiple strategies that can be used to overcome preexisting immunity. The epitopes of these nAb for AAV8 have been identified [50], and at least for AAV2, amino acid substitutions at epitopes have successfully conferred escape from a majority, but not all, of nAbs [51]. Another approach is to create a new AAV variant that is resistant to neutralization by preexisting antibodies through error-prone PCR in a technique called directed evolution of AAV vectors [52, 53]. However, it is also possible to minimize contact of the AAV vector to nAb by directly injecting the vector

into the target tissue rather than via circulation. It has been demonstrated that there was minimal impact on systemic gene expression from an AAV8 vector that was injected directly into muscle in non-human primates with preexisting AAV8 nAbs as high as 1/320 [54]. This has also been demonstrated in AAV1- and AAV2-mediated gene therapy clinical trials [41, 43], suggesting that intramuscular administration of AAV8-VIP, even in the presence of nAb, will yield in strong transgene expression.

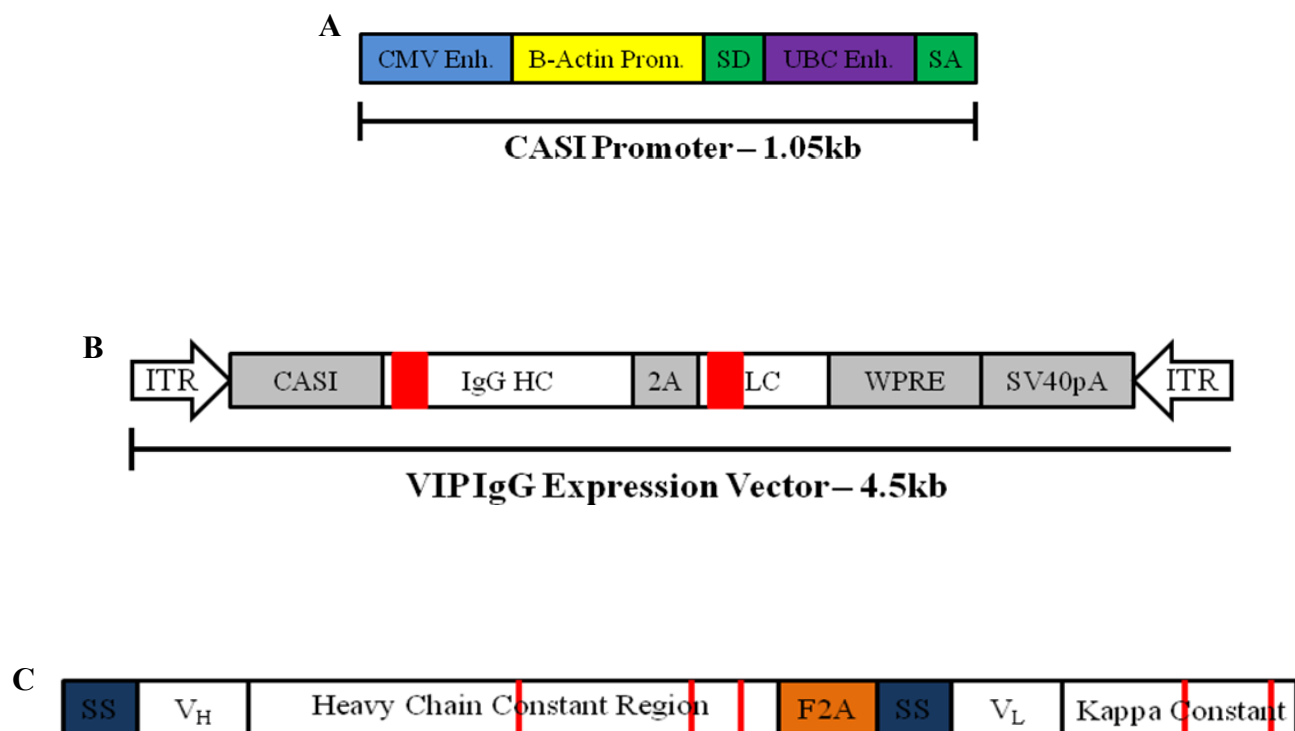
The use of VIP technology for malaria control depends upon long-term mAb expression. Clinical studies using AAV to target immunoprivileged sites such as retinal tissue [55] have shown remarkable success although transduction of the liver has induced an adaptive immune response against the vector capsid [42]. Studies have demonstrated that activation of T cells against the vector capsid is limited to serotypes that have a heparin binding motif and exhibit heparin-binding activity [27]. Interestingly, serotypes such as AAV8 that lack this activity are better tolerated *in vivo* since they do not induce cytotoxic T lymphocyte responses, making it more likely that long term transduction will be achieved. The route of administration of AAV also appears to impact the longevity of transgene expression as intramuscular transduction has been shown to yield in long-lived expression of factor IX, which is in contrast to liver transduction [40, 43, 56]. Although we only look at mAb expression out to 36 weeks, it has been previously demonstrated that expression is stable out to at least 72 weeks [28, 29], suggesting that this approach would result in long-term mAb expression necessary for an effective malaria control.

While the lack of a mouse-mediated immune response against the transgene is surprising, this is consistent with other AAV literature. Less than half of the mice developed a detectable antibody response against the human Fc region which did not appear to impact transgene expression and there was no indication of immune complex deposition upon pathological examination of mouse tissue (Figure 9). It is possible that the persistent high expression levels of human IgG antibody induced B cell anergy or exhaustion, a phenomenon that has been associated with autoimmune diseases, HIV and TB.



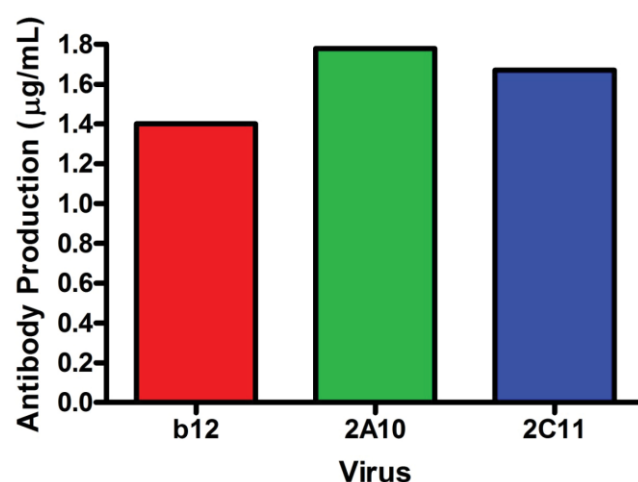
AAV vectors have been utilized for many years in both vaccine and gene therapy trials and have exhibited an exemplary safety record [22]. In our experiments, adverse events such as pathology from high titer human IgG expression were not detected. However, safety concerns still exist. A major concern centers on the ability of WT AAV to integrate into host DNA. Many studies have been undertaken to determine the frequency and general location of AAV integration events, both in WT infections and in gene therapy trials [57-63]. The level of integration, with WT AAV, is much lower than that of retroviral vectors [58]. In AAV vectors, like the one used in this study, that lack the *Rep* genes necessary for integration, the frequency of these events is closer to the number of random DNA integration events from DNA vaccines. Most studies found AAV to primarily exist as a circular extrachromosomal episome, with integration events being rare but happening more often in liver transduction than muscle [58, 59, 61, 62]. To date, no detected AAV integration has been associated with an increase in tumor formation or an increase in gene transcription [57, 59, 60]. Recently, the first AAV-vectored gene therapy has been approved in Europe for treating LPLD [23, 57]. Thus, the safety and long-term effects of AAV vectored therapies will continue to be investigated. More information on the use of VIP in humans will become available as Dr. Baltimore's laboratory is pursuing entering clinical trials in HIV infected individuals with their b12-AAV construct.

Despite decades of research, a malaria vaccine still does not exist and according to the WHO, in order to eradicate malaria, a vaccine would need to have 80% efficacy lasting for 4 years or longer. Given the urgency of combating malaria and inability of current vaccine candidates to induce effective long-lasting immunity, novel methods need to be explored. Our work demonstrates the feasibility of translating existing broadly neutralizing antibodies into a functional immunoprophylaxis *in vivo* that could work as a form of malaria control. The level of protection that VIP has demonstrated in our initial experiments indicates that this strategy could be optimized to combat malaria in endemic regions, with the end goal of eradication.



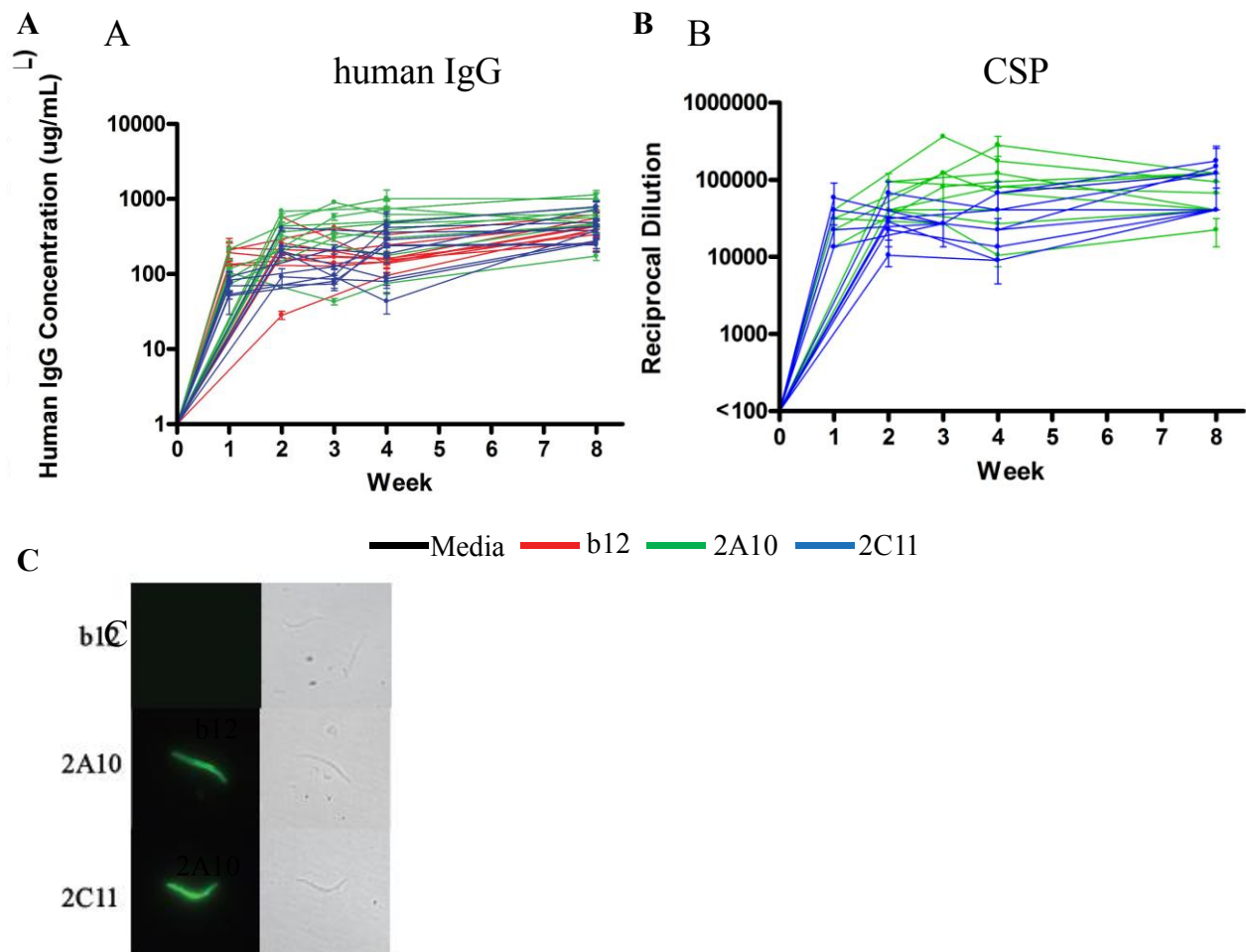
**Figure 1: Schematic representation of VIP expression vector.**

(A) The novel CASI promoter combines the cytomegalovirus (CMV) enhancer and chicken  $\beta$ -actin promoter followed by a splice donor (SD) and splice acceptor (SA) flanking the ubiquitin (UBC) enhancer region. (B) The VIP expression vector for antibody expression indicating the AAV2 inverted terminal repeats (ITR), the CASI promoter, an IgG<sub>1</sub> heavy chain linked to the light chain separated by a self-processing 2A sequence, a woodchuck hepatitis posttranscriptional regulatory element (WPRE) and an SV40 polyadenylation signal (SV40pA). Antibody variable regions of the heavy and light chains are colored in red (C) Schematic representation of the IgG<sub>1</sub> transgene that was optimized for expression in vitro. Highlighted in blue is the human growth hormone (HGH) derived signal sequence (SS) and the F2A self-processing peptide (orange), yielding in separate heavy and light chains. Red lines mark the predicted splice donor and acceptor sites that have been mutated.

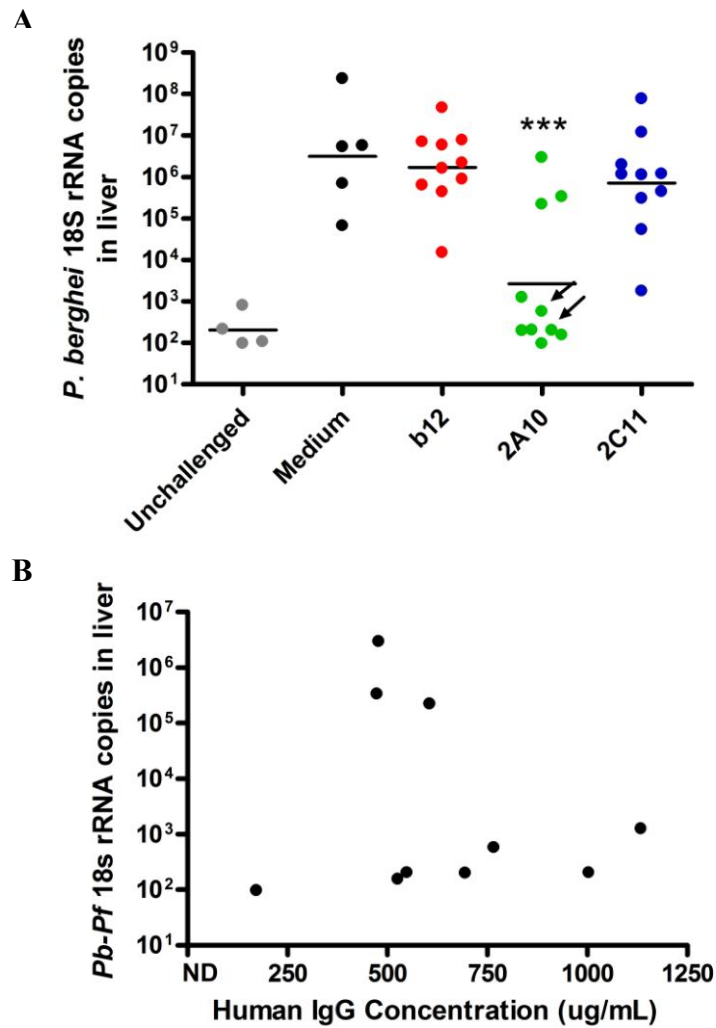


**Figure 2: AAV2/8 vectors express high quantity of human IgG antibodies *in vitro*.**

The functional activity of each VIP vector was tested *in vitro* by infecting 293T cells with AAV2/8 vectors expressing monoclonal antibody b12, 2A10 or 2C11. B12-AAV served as an expression control. Supernatant from infected cells was assayed for human IgG 6 days post infection by ELISA. Creation and *in vitro* characterization of these VIP vectors was performed by Dr. Alex Balazs.

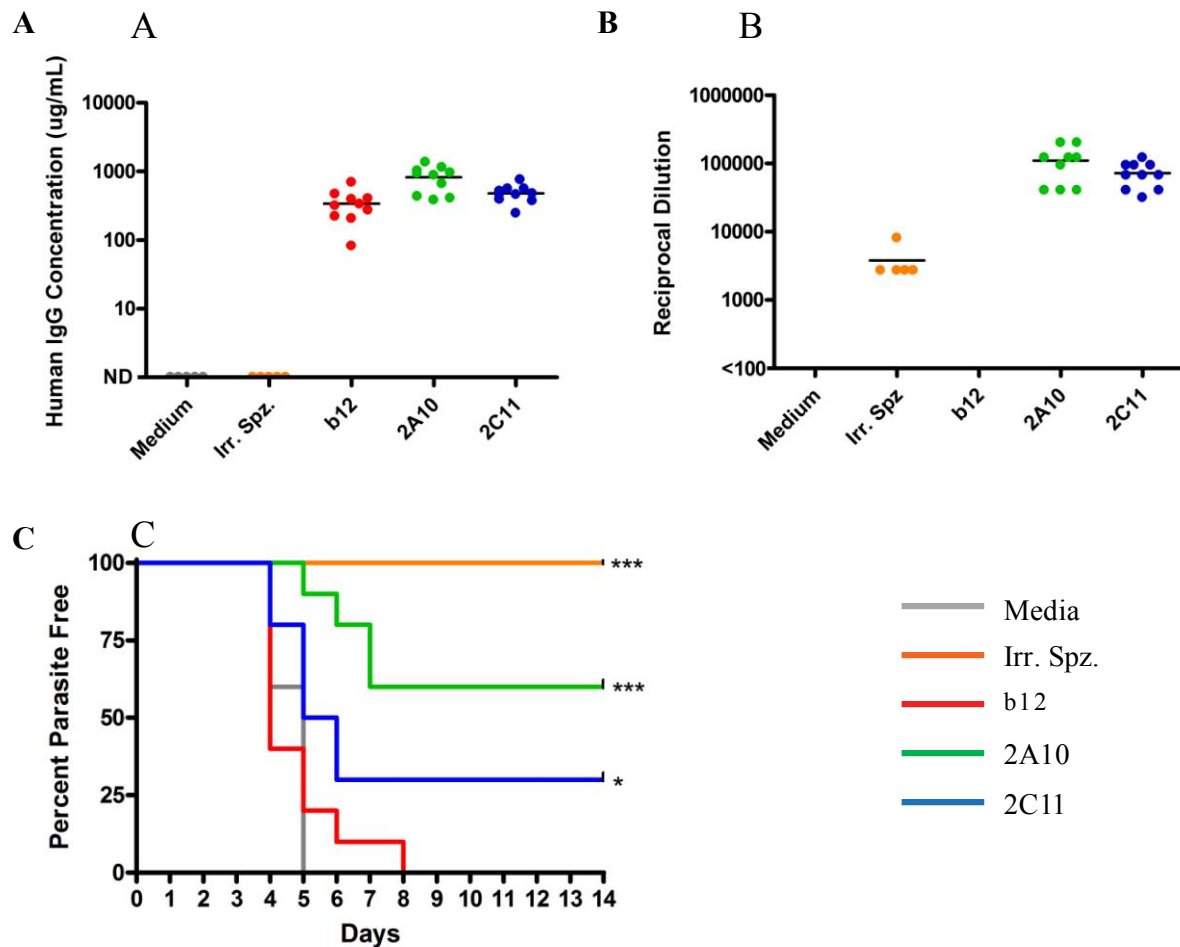


**Figure 3: All vectors express high levels of sustained human IgG antibody *in vivo*, and VIP-produced *P. falciparum* neutralizing antibodies retain ability to recognize CSP.** Quantification of human IgG (A) and anti-CSP antibodies (B) by ELISA after intramuscular injection of  $1 \times 10^{11}$  genome copies of the VIP expression vector producing b12, 2A10 or 2C11 in female C57BL/6. The plot shows mean and standard error of titers for each mouse,  $n=10$ . Sera from b12 transduced mice did not recognize recombinant CSP and thus is not shown in (B). (C) Immunofluorescence images of transgenic *Pb-Pf* sporozoites incubated with 500ng of human IgG from pooled sera from either b12-, 2A10- or 2C11-transduced mice. Phase-contrast images show the presence of sporozoites in the field.



**Figure 4: VIP administration significantly reduces parasite burden in the liver.**

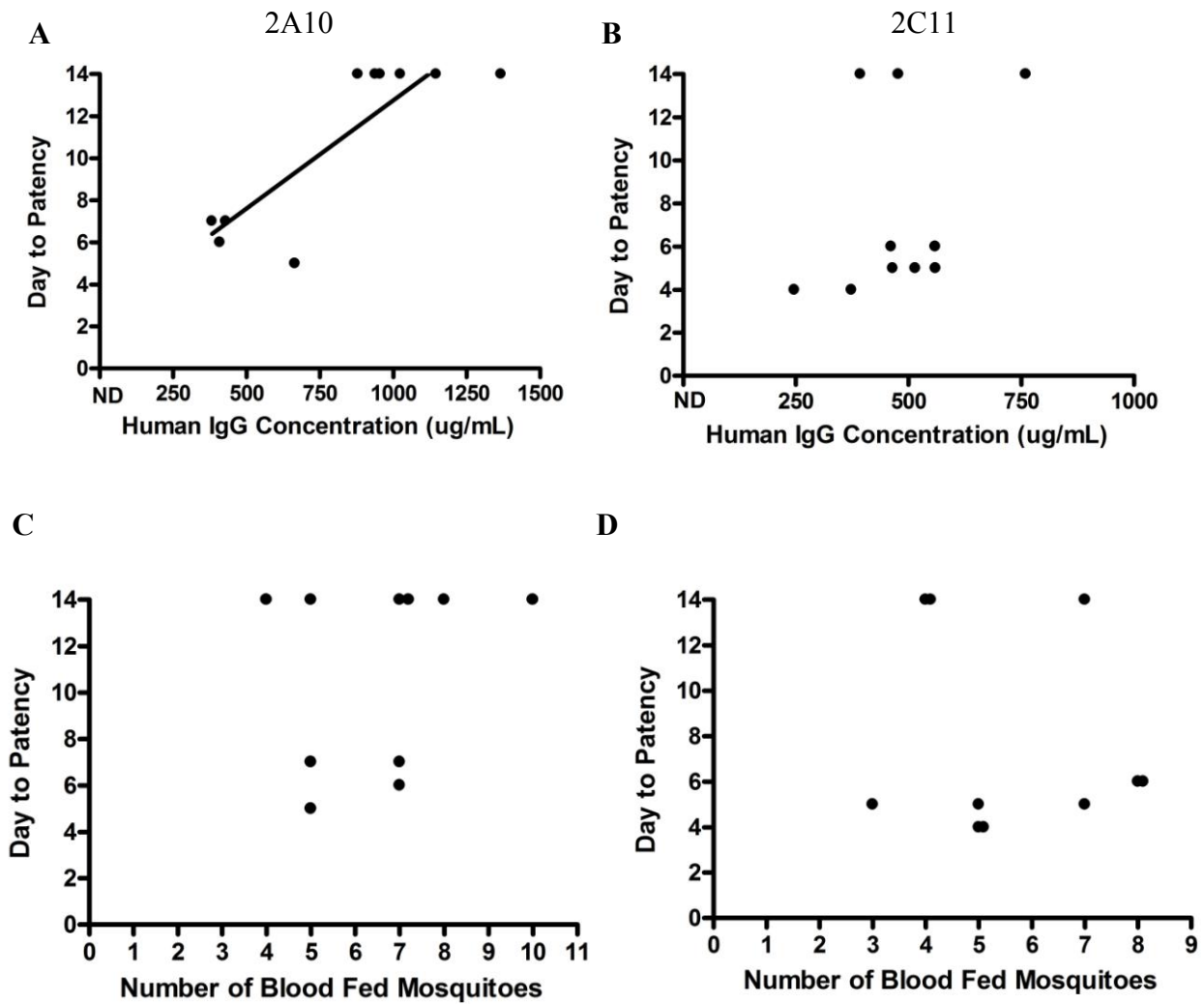
(A) Eight weeks post VIP administration, mice were challenged intravenously with  $1.0 \times 10^4$  *Pb-Pf* sporozoites. Parasite burden was assessed 40-42 hours post challenge on liver homogenates by qRT-PCR for *P. berghei* 18S rRNA copies. The plot shows values for each individual mouse and the geometric mean of the group (n=5-10). Arrows denote mice having a detectable anti-human Fc humoral immune response. \*\*\*p<0.001. (B) Correlation between liver parasite burden 40-42 hours post challenge and human IgG serum concentration for 2A10-transduced mice.



**Figure 5: VIP can provide sterile protection to mice challenged by infected mosquito bite.**

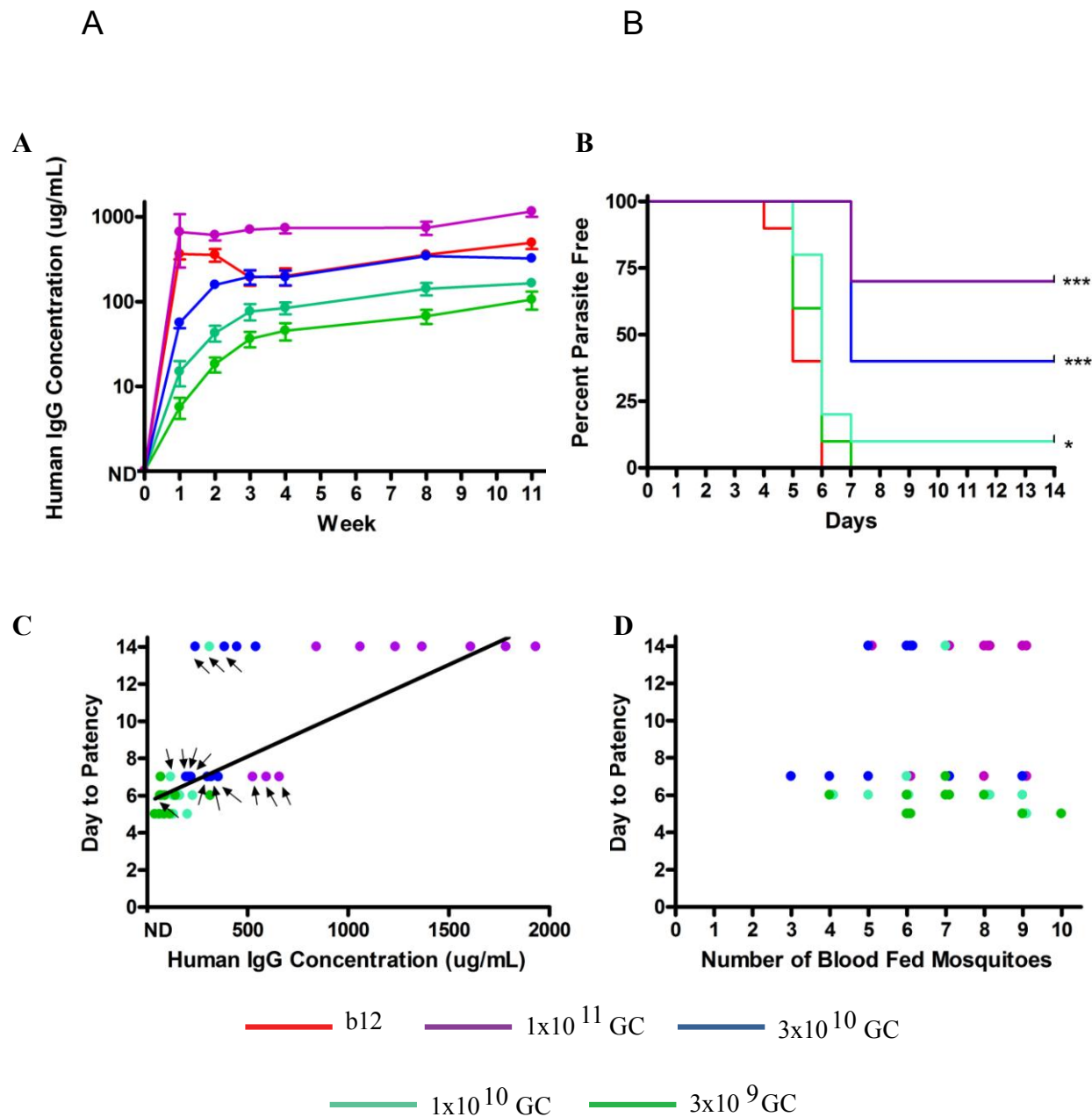
Concentration of human IgG (A) and anti-CSP (B) antibodies measured by ELISA on serum samples either taken 11 weeks after intramuscular injection of media or vector expressing b12, 2A10 or 2C11, or taken 3 weeks after the third injection of  $1.0 \times 10^5$  to  $1.5 \times 10^5$  irradiated *Pb-Pf* sporozoites. (C) Mice that received VIP 11 weeks prior or 3 weeks after the third dose of irradiated *Pb-Pf* sporozoites were challenged with *Pb-Pf* sporozoites delivered by infected mosquito bite. The Kaplan Meier survival curve depicts percent of mice that were parasite-free, determined by monitoring mice for the presence of blood-stage parasites in blood smears taken daily beginning at 4 days post challenge (n=10). \*p<0.05

\*\*\*p<0.005



**Figure 6: Sterile protection correlates with human IgG concentration in sera prior to challenge in 2A10-transduced mice.**

Correlation between days to patency and serum human IgG concentration (A, B) or number of mosquito bites (C, D) for 2A10- (A, C) and 2C11-transduced (B, D) mice. Plot shows values for each individual mouse. Linear regression is shown on (A) to help visualize the correlation.

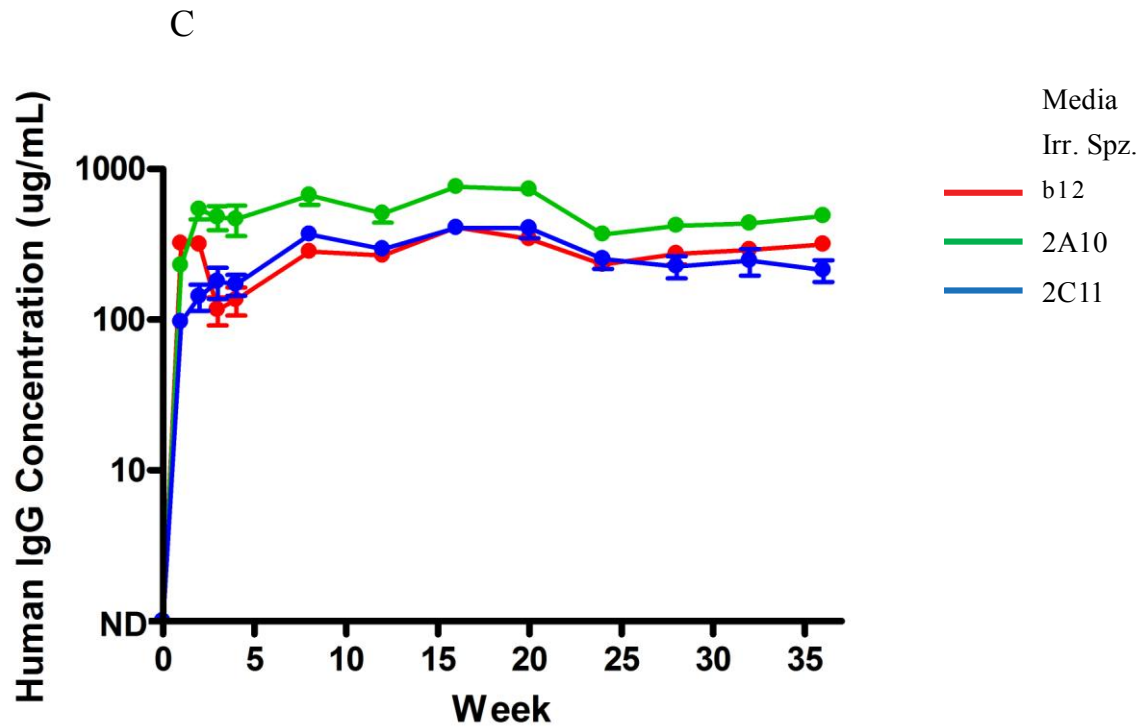


**Figure 7: Antibody expression and protection are dependent on VIP vector dose.**

(A) 2A10 expression over time as a function of vector dose as determined by total human IgG ELISA on serum samples taken after AAV administration. B12-denoted mice received  $1 \times 10^{11}$  genome copies of b12-expressing vector. Plot shows mean and standard error (n=10). (B) Mice were challenged 11 weeks after VIP-administration with *Pb-Pf* sporozoites by infected mosquito bite. Survival curve depicts the percentage of mice that do not show blood-stage parasites. \*\*\*p<0.005 \*p<0.05. (C, D) Correlation between days to patency and serum human IgG concentration (C) or number of mosquito bites (D) for



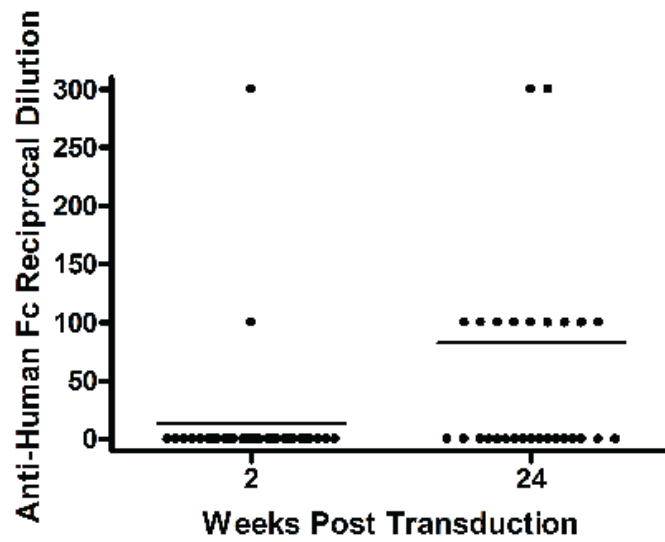
transduced mice. Arrows denote mice with a detectable anti-human Fc antibody response. Linear regression is shown in (C) to help visualize the correlation.



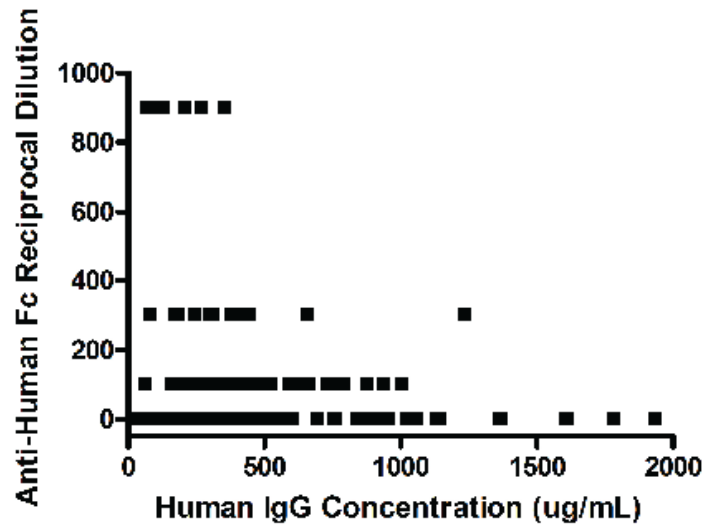
**Figure 8: Expression of human IgG by VIP is sustained over time *in vivo***

Quantification of human IgG by ELISA in sera of mice transduced with b12-, 2A10 or 2C11-expressing vectors. Plot shows mean and standard error (n=10)

A



B



**Figure 9: Anti-transgene immune response increases over time but does not affect human IgG concentration**

(A) Anti-human Fc reciprocal dilution titers for week 2 and week 24 post transduction as determined by ELISA. (B) Correlation between levels of detectable anti-human Fc antibodies, measured by ELISA, and

human IgG concentration in sera. Plot depicts anti-human Fc antibody levels at the last bleed before challenge for all VIP transduced mice ( $p=0.5538$ ,  $r=-0.04657$ ).

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# Cailin Deal

## Work Address

Johns Hopkins University  
Bloomberg School of Public Health  
615 N. Wolfe Street  
Baltimore, MD 21205  
Telephone: (410) 502-2521

## Home Address

26 N. Kenwood Ave  
Baltimore, MD 21224  
Cell: (860) 204-1123  
Email: cailindeal814@gmail.com

## EDUCATION

**Johns Hopkins Bloomberg School of Public Health** 2008-present

- Ph.D candidate in Molecular Microbiology and Immunology
- Thesis: Conserved epitopes in *Plasmodium falciparum* and influenza A virus as targets for virus-vectored immunization
- Laboratories of Dr. Gary Ketner and Dr. Andrew Pekosz
- Anticipated date of completion: January 2014

**University of Connecticut** 2004-2008

- Bachelor of Science in Molecular Cell Biology (*summa cum laude*)

## RESEARCH EXPERIENCE

*Ph.D Candidate*, **Johns Hopkins Bloomberg School of Public Health** 2008-present

- Use of adeno-associated virus expressing full-length monoclonal human antibodies to confer immunity to *P. falciparum* infection (in collaboration with Dr. David Baltimore, California Institute of Technology)
- Investigation of the role of the highly conserved M2 ectodomain in the influenza A life cycle through scanning alanine mutagenesis and transcomplementation assays
- Investigation of adenovirus vectors displaying the M2 ectodomain and HA2A helix of influenza A virus on the virion surface as 'universal' multivalent influenza vaccines

*Intern*, **Red Cross Childrens' War Memorial Hospital in Cape Town** 2008

- Conducted retrospective case research on the incidence of ventilated associated pneumonia in burn victims
- Interviewed patients families for information on burn incident in concurrent patients with ventilated associated pneumonia

*Intern*, **Pfizer Inc. Protein and Cell Sciences Department** 2007

- Conducted an array synthesis of novel derivatives of commercial peptides as a target for drug treatment for obesity

*Research Assistant, University of Connecticut* 2006-2007

- Laboratory of Dr. Kenneth Noll
- Investigated the use of *Escherichia coli* NADH dehydrogenase mutants as a catalyst for a microbial fuel cell

*Research Assistant, University of Connecticut* 2005-2006

- Laboratory of Dr. Harry Frank
- Purified and analyzed photosynthetic pigments from different algal species using HPLC and UV/Vis spectrometry
- Performed ultrafast time-resolved transient absorption spectroscopic studies of carotenoid monomers from dark-adapted and illuminated thylakoids

*Intern, Pfizer Inc. Cardiovascular and Metabolic Diseases Department* 2005

- Extracted cellular metabolites from isolated peripheral blood leukocytes
- Developed a capillary electrophoresis method for the analysis of cellular metabolites

## TEACHING EXPERIENCE

*Teaching Assistant, Johns Hopkins Bloomberg School of Public Health*

- Introduction to Biomedical Sciences 2012
- Graduate Immunology 2012
- Principles of Bacteriology 2009

## HONORS AND AWARDS

- Johns Hopkins School of Public Health (JHSPH) Alfred Sommer Scholar 2010- present
- JHSPH Vaccine Day Poster Competition – 2<sup>nd</sup> place 2011
- American Society for Virology Student Travel Award 2011
- JHSPH Vaccine Day Poster Competition – 3<sup>rd</sup> place 2010
- JHSPH Keerti V. Shah Fellow 2009
- UConn Honors in Cape Town (internship honors study abroad) 2008
- UConn Bobbitt Internship (undergraduate chemistry research) 2005
- University of Connecticut Presidential Scholar Award 2004

## PUBLICATIONS

- Deal C, Pekosz A, Ketner G. Prospects for Oral Replicating Adenovirus-Vectored Vaccines. *Vaccine*. 2013; 31(32): 3236-43.
- Galinato MGI, Niedzwiedzki D, Deal C, Birge RR, Frank HA. Cation radicals of xanthophylls. *Photosynth Res*. 2007; 94:67-78.

## PRESENTATIONS

- **Deal C**, Balazs AB, Zavala F, Baltimore D, Ketner G. 2013. Antibody-based vectored immunoprophylaxis provides high-titer *Plasmodium falciparum* circumsporozoite antibodies. World Malaria Day 2013, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland.
- **Deal C**, Ketner G, Pekosz A. 2011. The Role of the Influenza A Virus M2 Protein Extracellular Domain in Virus Replication and on the Induction of Broadly Protective Antibodies. Oral presentation at American Society for Virology 30<sup>th</sup> Annual Meeting, Minneapolis, Minnesota
- **Deal, C.**, Ketner G., Pekosz A. 2010. Development of a Multivalent ‘Universal’ Influenza Vaccine by Utilizing Capsid Display Recombinant Adenoviruses Expressing Highly Conserved Epitopes of M2 and Hemagglutinin. Vaccine Day Poster Competition, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland.

#### **AFFILIATIONS**

- American Society for Microbiology
- American Society of Virology

#### **PROFESSIONAL SERVICE**

- Journal Reviewer for Vaccine
- *Ad Hoc* Journal Reviewer for PLOS Pathogens

#### **RESEARCH INTERESTS**

- Vaccine development
- Virus vector technology
- Emerging infectious diseases
- Host-virus interactions